(71) Applicant (for all designated States except US): CRU-CELL HOLLAND B.V. [NL/NL]; Archimedesweg 4, NL-2333 CN Leiden (NL).

(72) Inventors; and
(75) Inventors/Applicants (for US only): HOUTZAGER, Erwin [NL/NL]; Koenestraat 13, NL-3958 XD Amerongen (NL). LOGTENBERG, Ton [NL/NL]; Dorpsstraat 19, NL-3433 CH Nieuwegein (NL). DE KRUIF, Cornelis, Adriaan [NL/NL]; Zilvergeldstraat 11, NL-3513 VN Utrecht (NL).

(74) Agent: PRINS, A., W.; Nieuwe Parklaan 97, NL-2587 BN Den Haag (NL).

(54) Title: CHIMAERIC PHAGES

(57) Abstract: The invention relates to the field of generating helper phages and phage display libraries for the identification of binding molecules. The invention provides chimaeric phages having a coat comprising a protein mixture comprising a fusion protein wherein a proteinaceous molecule is fused to a functional form of a phage coat protein and a mutant form of the phage coat protein, the mutant form being impaired in binding to a host cell receptor. The invention further provides new phage collections, novel helper phages and methods and means for producing chimaeric phages, infectious phages and helper phages according to the invention.

Title: Chimaeric phages

FIELD OF THE INVENTION

5   The present invention relates to the fields of
molecular biology and immunology. The invention in
particular relates to the field of generating helper
phages and phage display libraries for the identification
of binding molecules.

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BACKGROUND OF THE INVENTION

        An individual needs to have a dynamic immune system
that is able to adapt rapidly to respond adequately to
15   potentially harmful microorganisms and to respond to the
exposure of a highly diverse and continuously changing
environment. Higher organisms have evolved specialized
molecular mechanisms to ensure the implementation of
clonally-distributed, highly diverse repertoires of
20   antigen-receptor molecules expressed by cells of the
immune system: immunoglobulin (Ig) molecules on B
lymphocytes and T cell receptors on T lymphocytes. A
primary repertoire of (generally low affinity) Ig
receptors is established during B cell differentiation in
25   the bone marrow as a result of rearrangement of germ line-
encoded gene segments. Further refinement of Ig receptor
specificity and affinity occurs in peripheral lymphoid
organs where antigen-stimulated B lymphocytes activate a
somatic hypermutation machinery that specifically targets
30   the immunoglobulin variable (V) regions. During this
process, B cell clones with mutant Ig receptors of higher
affinity for the inciting antigen are stimulated into
clonal proliferation and maturation into antibody-
secreting plasma cells (reviewed in Berek and Milstein.
35   1987).

Recombinant DNA technology has been used to mimic many aspects of the processes that govern the generation and selection of natural human antibody repertoires (reviewed in Winter and Milstein. 1991; Vaughan et al.

5   1998). The construction of large repertoires of antibody fragments (such as Fab fragments or single chain Fv fragments, scFv's) expressed on the surface of filamentous phage particles and the selection of such phages by "panning" on antigens has been developed as a versatile

10   and rapid method to obtain antibodies of desired specificities (reviewed in Burton and Barbas. 1994). A subsequent optimization of the affinity of individual phage antibodies was achieved by creating mutant antibody repertoires of the selected phages and sampled for higher

15   affinity descendents by selection for binding to antigen under more stringent conditions (reviewed in Hoogenboom. 1994).

M13 and M13-derived phages (sometimes also called viruses) are filamentous phages that can selectively

20   infect F-pili bearing (F^+) *Escherichia coli* (*E.coli*) cells. The phage genome encodes 11 proteins, while the phage coat itself consists of 5 of these proteins: gene3, -6, -7, -8 and -9 (g3, g6, g7, g8 and g9) proteins that are bound to and that protect the (circular) single

25   stranded DNA (ssDNA) of the viral genome. The life cycle of the virus can be subdivided into different phases. The g3 protein (g3p) of M13 phages and M13-derivatives comprises three functional domains: D1, D2 and D3, linked by two glycin-rich linkers. An alternative nomenclature

30   for g3p domains has also been generally accepted, in which D1, D2 and D3 are named N1, N2 and CT, respectively. The N-terminal D1/D2 regions interact with the C-terminal D3 region as has been found by Chatellier et al. (1999) using several deletion mutants of g3p. Considering that

35   functionality of a D3 domain of the protein is required for assembly of stable phages,

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a less-, or non-infectious mutant of said phage coat
protein preferably comprises a D3 region of said g3p, or
comprises a functional part, derivative and/or analogue of
said D3 region. The D3 domain is thought to bind to DNA

5   inside the viral particle. Loss of the D3 domain
functionally results in rare phage-like particles that are
very long and very fragile (Pratt et al. 1969; Crissman
and Smith. 1984; Rakonjac and Model. 1998). The D1 and D2
domain are thought to interact with each other until the

10   phage binds to the bacteria, while D1/D2 also interact
with D3 at certain stages (Chatellier et al. 1999). The
linkers present in g3p between D1, D2 and D3 apparently
also play a role in infectivity of the phage particle
(Nilsson et al. 2000). Studies in which a protease

15   cleavage site was introduced between D1 and D2 showed that
after cleavage the phage particle became non-infectious
(Kristensen and Winter. 1998). Functional analysis of g3p
showed that of the g3p N-terminal regions, the D1 domain
is essential for infection. Loss of this domain results in

20   phages that cannot infect bacteria (Lubkowski et al. 1998;
Nelson et al. 1981; Deng et al. 1999; Riechmann and
Holliger. 1997; Holliger and Riechmann. 1997). It has been
shown that the D2 domain interacts with the D1 domain of
g3p on the phage (Figure 1). Due to competition of

25   proteins located on the F-pilus (on F$^+$ bacteria) that have
higher affinity for D2 than for D1, the D1 and D2 domain
of the g3p dissociate from each other. The binding of D2
to the F-pilus results in a process that leads to
retraction of the F-pilus towards the E.coli cell

30   membrane. Due to this process the phage particle comes in
close contact with the bacterial membrane. Now the
dissociated D1 domain can interact with bacterial proteins
such as the TolA receptor, leading to the introduction of
the phage DNA into the E.coli cell (Lubkowski et al.

35   1999). The fact that removal of the D2 domain does not
prevent infection but enables phages to infect E.coli

lacking F-pili (Riechmann and Holliger. 1997; Deng et al.
1999) shows that the presence of the D2 domain increases
specificity and that D2 has an important role in
preventing F-pili independent infections. The binding of
5   D1 to the specific receptors on the surface of the *E.coli*
cell (a feature that is not F$^+$-specific) is represented in
Figure 2. This process triggers the injection of the viral
genome into the bacterium (as depicted in Figure 3).
Although loss of the D2 domain results in the formation of
10   phage particles that can infect *E.coli* in a somewhat
reduced specific manner, it appears that the level of
infections from such a population of phages is
significantly reduced. After infection of an *E.coli* by a
phage particle, the ssDNA of the virus becomes double
15   stranded due to the action of a number of bacterial
enzymes. The double stranded phage genome now serves as a
template for the transcription and translation of all 11
genes located on the phage genome. Besides these protein-
encoding regions, the phage genome contains an intergenic
20   region: the F1-origin of replication initiation (F1-ORI).
The DNA sequence of this F1-ORI can be divided in 2
separate subregions. One subregion is responsible for the
initiation and termination of the synthesis of ssDNA via
the so-called 'rolling circle mechanism' and the other
25   subregion is responsible for the packaging initiation of
the formed circular ssDNA leading to the formation and
release of new virus particles.

It has been shown that polypeptides, such as
stretches of amino acids, protein parts or even entire
30   proteins can be added by means of molecular genetics to
the terminal ends of a number of particle coat proteins,
without disturbing the functionality of these proteins in
the phage life cycle (Smith. 1985; Cwirla et al. 1990;
Devlin et al. 1990; Bass et al. 1990; Felici et al. 1993;
35   Luzzago et al. 1993).

This feature enables investigators to display
peptides or proteins on phages, resulting in the
generation of peptide- or protein expression phage display
libraries. One of the proteins that has been used in the
5   art to fuse with polypeptides for phage display purposes,
is the g3 protein (g3p), which is a coat protein that is
required for an efficient and effective infectivity and
subsequent entry of the viral genome into the E.coli cell.
     For the production of phages that display
10   polypeptides fused to the g3p coat protein, investigators
in the art introduced a plasmid together with the phage
genome in E.coli cells. This plasmid contains an active
promoter upstream of an in-frame fusion between the g3
encoding gene and a gene of interest (X) encoding, for
15   instance, polypeptides such as proteins such as antibodies
or fragments such as Fab fragments or scFv's. The
introduction of this plasmid together with the genome of
the helper phage in an E.coli cell results in the
generation of phages that contain on their coat either the
20   wild type g3p from the viral genome, the fusion product
g3p-X from the plasmid or a mixture of the two, since one
phage particle carries five g3p's on its surface. The
process of g3p or g3p-X incorporation is generally random.
The presence of an F1-ORI sequence in the g3p-X expression
25   vector (plasmid) misleads the phage synthesis machinery in
such a way that two types of circular ssDNA are formed:
one is derived from the genome of the phage and the other
is derived from the expression vector. During the
synthesis of new phages the machinery is unable to
30   distinguish the difference between these two forms of
ssDNA resulting in the synthesis of a mixed population of
phages, one part containing the phage genome and one part
harbouring the vector DNA. Due to these processes the
mixture contains at least some phages in which the
35   phenotypic information on the outside (the g3p-X fusion
protein) is conserved within the genotypic information

inside the particle (the g3p-X expression vector). An
infectious wild type phage and a phage carrying a fusion
protein attached to g3p are depicted in Figure 4. The art
teaches that there are several problems that concern the
5   use of these basic set-ups.

The high level of genotypic wild type phages in phage
populations grown in bacteria that contain both the phage
genome and the expression vector compelled investigators
to design mutant F1-ORI sequences in M13 genomes. Such
10 mutant M13-strains are less effective in incorporating
their genome in phage particles during phage assembly,
resulting in an increased percentage of phages containing
vector sequences when co-expressed. These mutant phages,
such as the commercially available strains R408, VCSM13
15 and M13KO7, are called "helper phages". The genome of
these helper phages may contain genes required to assemble
new (helper-) phages in *E.coli* and to subsequently infect
new F-pili expressing *E.coli*. Both VCSM13 and M13KO7 were
provided with an origin of replication initiation (ORI) of
20 the P15A type that results in the multiplication of the
viral genome in *E.coli*. Moreover, the ORI introduction
ensures that after cell division the old and newly formed
E.coli contains at least one copy of the viral genome.

It was suggested and finally proven by several
25 investigators that the introduction of plasmids containing
a g3p-scFv fusion product together with the genome of the
helper phages in *E.coli* cells results in approximately 99%
of newly formed phages that harbor the g3p-scFv fusion
protein expression plasmid but nevertheless lack the g3p-
30 scFv fusion on its surface (Beekwilder et al. 1999). The
absence of g3p-X is a significant disadvantage in the use
of display libraries for the identification of specific
proteins or peptides such as scFv's that bind to a target
of interest (such as tumor antigens). It implies that in
35 the case of phage display libraries at least a 100-fold
excess of produced phages must be used in an experiment in

order to perform a selection with all possible fusion
proteins present. The art teaches that this overload of
relatively useless phages in an experiment leads to (too)
many false positives. For instance, at least 10^{12} phages

5   should be added to a panning experiment in order to have 1
copy of each possible fusion present in the experiment,
since such a library contains approximately 10^{10} different
g3p-scFv fusions (1%). The phages in this approximate 1%
express generally only 1 g3p-scFv fusion on their coat

10 together with four normal g3p's (no fusions), while the
rest of the helper phages (approximately 99%) express five
g3p's and no g3p-scFv fusions. To ensure, theoretically,
the presence of 100 copies of each separate fusion protein
in a panning experiment, one needs to use approximately

15 10^{14} phages in such an experiment. Persons skilled in the
art generally attempt to use an excess of at least 100-
fold of each single unique fusion protein, to ensure the
presence of sufficient numbers of each separate fusion and
not to lose relevant binders too quickly in first panning

20 rounds. That number of phages (10^{14}) is more or less the
maximum of phage particles that a milliliter (ml) can
hold. The viscosity of such a solution is extremely high
and therefore relatively useless. Especially when ELISA
panning strategies are used (in which the volume of one

25 well is only 200 µl) such libraries cannot be used.
        In addition to these problems, it is assumed that
generally, depending on the antigen and the stringency of
washing procedures, an average of 1 in every 10^7 phages
will bind to the antigen due to a-specific binding. Thus,

30 for the application of 10^{12} scFv expressing input phages
(1%) to a panning procedure, one has to add approximately
10^{14} phages (99% of which do not express a scFv fragment).
It is generally assumed that from these 10^{12} phages
approximately 10^4 particles might be putatively

35 interesting phages. However, depending on the washing
conditions, the number of calculated background phages

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that are normally found by using libraries present in the
art after one round of panning, was approximately 10^6-10^7
while only a few of these phages appear to be relevant
binders. This is one of the most significant problems

5   recognized in the art: too many background phages show up
as initial binders in the phage mix after the first round
of panning, while only a few significant and interesting
binders are present in this mix. So, the absolute number
of isolated phages after one round of panning is clearly

10   too high (10^6-10^7). Moreover, also in subsequent rounds of
panning non-specific background phages remain present. In
libraries used in the art, most of these non-specific
binders will amplify on bacteria upon amplification to
continue appearing in a second round of panning.

15   Therefore, the art teaches that the background level of a-
specifically binding phages and the total number of phages
per ml in these types of libraries is unacceptably high
and remains high during subsequent rounds of panning.

A possibility that was suggested by investigators in

20   the art as a solution to the problem of obtaining too many
background phages that lack a g3p-X fusion was to remove
the g3p-encoding gene entirely from the helper phage
genome. In principle, this system ensures that during
phage synthesis in an *E.coli* cell (that received the g3-

25   less phage genome and a g3p-X fusion protein expression
vector) only g3p-X proteins are incorporated in the newly
formed phage coat. By doing so, each synthesized phage
will express five copies of the g3p-X fusion product and
hardly any phages are synthesized that express the g3p

30   alone or that express less than five g3p-X fusions. R408-
d3 and M13MDΔD3 are two examples of g3-minus helper phages
(Dueñas and Borrebaeck. 1995; Rakonjac et al. 1997).
Because the genome of these phages is not capable of
supporting g3p synthesis, phage particles that carry less

35   than five g3p-X fusion proteins can hardly be formed, or,
if formed, are found to be non-infectious due to

instability, since the art teaches that five g3p's are
necessary to ensure a stable phage particle.

To produce helper phages that do not contain the g3
gene, but that are nevertheless infectious and that can be
5   used to generate libraries of phages that carry five g3p-X
fusion proteins, and that lack phages with less than five
g3p-X fusions, it was recognized in the art that an
external source for g3p was required. Such a source can be
a vector without F1-ORI but that nevertheless contains an
10   active promoter upstream of the full open reading frame
(ORF) of g3. One major problem that is recognized by
persons skilled in the art is that after the generation
step of producing newly formed helper phages lacking a g3
gene, the yield is dramatically low. In fact, the yield of
15   all described systems is below 10^{10} phages per liter,
meaning that for a library of 10^{10} individual clones at
least 100 liter of helper phage culture is necessary (NB:
the helper phages need to be purified) in order to grow
the library once. The art thus teaches that phage
20   libraries generated with such low titers of helper phages
are not useful for phage display purposes and that
therefore these libraries cannot be used for panning
experiments. One method for complementation of g3p
deletion phages was presented recently, in which wild-type
25   g3p was provided by a nucleic acid encoding the wild-type
g3p, wherein the nucleic acid was stably incorporated into
the host cell genome (Rondot et al. 2001).

Phages that express deleted g3p's fused to
heterologous proteins have been generated. For the
30   construction of most conventional Fab libraries and some
scFv phage display libraries the D1 domain and parts of
the D2 domain were removed to ensure a shorter fusion
protein, which was considered in the art as a product that
could be translated easier than a full length g3p linked
35   to a full length Fab fragment. The shorter g3p part would
not prevent the generation of a viable and useful helper

phage. Of course, such phages still depend on full length
g3p's that are present on their surface next to the
deleted g3p fusion with the Fab fragment for functional
infectivity of *E.coli* cells. Also, phages that express
5   deleted g3p's fused to ligand-binding proteins have been
generated that depend on their infectious abilities on
antigens that were fused to the parts of g3p that were
missing from the non-infectious phage particle (Krebber et
al. 1997; Spada et al. 1997). These particles depend for
10 their infectivity on an interaction between the ligand-
binding protein, such as an antibody or a fragment thereof
and their respective ligand (or antigen). However, this
interaction-dependency reduces the efficiency of
infection, due to elimination of a direct linkage between
15 the g3p domains, and a general inhibitory effect of the
soluble N-terminal part of g3p coupled to the antigen.

        The g3-minus helper phages R408-d3 and M13MDΔD3
mentioned above, lack in their genome a bacterial ORI and
a selection marker. The absence of a selection marker in
20 the g3-minus genomes has a significant effect on the
production scale of helper phages, because it results in
an overgrowth of bacteria that do not contain the helper
phage genome. It is known that bacteria grow slower when
infected with the helper phage or virus. Therefore,
25 bacteria that lack the phage genome quickly overgrow the
other bacteria that do contain the genome. Another effect
of the lack of an ORI or a selection marker is that g3-
minus phage genomes cannot be kept in dividing bacteria
during the production and expansion of phage display
30 libraries. This is a very important negative feature
because overgrowth of bacteria that lost the phage genome
or that did never receive one, appear to have a growth
advantage over bacteria that do contain the phage genome.
In addition of course, such 'empty' bacteria are not
35 capable of producing any phage and as a result of this the
phage display vectors including fusion protein fragments

in such helper phages lacking-bacteria are lost
permanently.

As mentioned, the g3p's are thought to be essential
for the assembly of stable M13-like phages and because of
their crucial role in infection, g3p's should be provided
otherwise when g3-minus helper phages are to be generated.
There is a prejudice in the art against making phage
display libraries that lack g3p's because phages lacking
g3p's are not stable. Rakonjac et al. (1997) constructed a
VCSM13 g3-minus helper phage in parallel to a R408 g3-
minus helper phage and used helper plasmids with either
the psp or the lac promoter upstream of a full length g3
sequence to substitute g3 during helper phage synthesis
(Model et al. 1997). However, the art teaches that the lac
promoter has the disadvantage that it cannot be shut off
completely, even not in the presence of high
concentrations of glucose (3-5%) in the medium (Rakonjac
and Model. 1998). An additional problem that is well known
in the art is that even very low levels of g3p in *E.coli*
can block infection of M13-like phages. Moreover, it has
been shown that co-encapsidation of plasmids together with
the phage genome can occur (Russel and Model. 1989;
Krebber et al. 1995; Rakonjac et al. 1997). If co-
encapsidation occurs with the lac driven helper plasmid it
will compete with the lac driven vectors used in the phage
display resulting in the efficient production of
infectious phage particles that will not contain the g3p-X
fusion product. Together, the art thus teaches that the
lac promoter is not the best candidate promoter in the
helper plasmid system. The psp promoter has the advantage
to be relatively silent in *E.coli* until infection
(Rakonjac et al. 1997). Upon M13-class phage infection the
psp promoter becomes activated and now the helper plasmid
will produce g3 proteins. However, the disadvantage of
this promoter is that the level of RNA production cannot
be regulated with external factors, but has to be

regulated by either mutating (and change the activity of)
the promotor, changing the ribosomal binding site (RBS) or
other elements that influence the promotor activity. To
figure out the ideal level of promotor activity in a

5   specific *E.coli* strain can be time consuming and needs to
be optimized for each *E.coli* strain separately. The art
also teaches that the psp promotor system is not very
attractive for large-scale helper phage production due to
the inflexibility of *E.coli* strains, the time consuming

10   optimization and the significant low level of helper phage
production.

A significant problematic feature of all helper phage
systems described is the occurrence of unwanted
recombination events between the helper genome and the

15   (helper-) plasmids. The problem that confronts
investigators in the art is the fact that the g3 DNA
sequences in the helper phages are homologous to the g3
sequences in the phage display vector and/or the helper
phage plasmid. This results in many cases in recombination

20   between the two DNA strains and therefore loss of
functionality of the library as a whole.

It is an object of the present invention to deal with
some of the problems and drawbacks known from the art as
described above, concerning the generation of phage

25   particles and helper phages, the use of helper phages in
the production of phage display libraries, and the
problems and drawbacks known for the identification of
relevant binding molecules using such libraries.

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SHORT DESCRIPTION OF THE FIGURES

Figure 1. Schematic representation of the g3 protein (g3p)
present in the coat of M13 phages. The D3 domain of g3p is
5   attached to the single stranded DNA inside the particle
via the g8 protein (g8p), while the D1 and D2 domains
interact with each other outside the particle and can be
used for fusion with for example scFv.

10   **Figure 2.** Schematic representation of the interaction
between the D2 domain of g3p with the F-pilus on the
surface of *E.coli*, with a subsequent interaction of the D1
domain with other components of the bacterial surface.

15   **Figure 3.** Schematic representation of the interaction of
the D2 domain of g3p with the F-pilus (left) and the D1
domain of g3p with the TolA receptor (see Figure 2) and
the subsequent entry (right) of the phage genome into the
cytoplasm of the bacterium.
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Figure 4. Schematic representation of a wild type phage
expressing five g3p's on its infectious end (left) and a
recombinant phage expressing four wild type g3p's and one
g3p-X fusion protein on its infectious end (right). The
25   recombinant phage also harbors the genetic information of
the fusion protein present on the surface.

Figure 5. Schematic representation of the pBAD/gIII-g3
helper vector harboring the full-length g3 gene under the
30   control of the AraC/BAD promoter and further harboring an
ampicillin resistance and a ColE1 origin of replication
(ORI).

Figure 6. (A) Schematic representation of the VCSM13
35   helper phage genome. The 11 genes are indicated, as well
as the kanamycin resistance gene (Kan R), the packaging

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signal (PS) and the large and small fragments of the original intergenic region (IG). (B) Sequence of the VCSM13 genome shown in (A). The translation of the g3 gene is given in one letter code. (C) Schematic representation

5   of the VCSM13-derived g3-minus helper phage genome deleted for the open reading frame (ORF) of the g3 gene. (D) Sequence of the part of the helper phage genome that surrounds the position of the g3 deletion depicted in (C). The HindIII site at position 3431 is underlined, followed

10   by the 6 codons in bold face upstream of the TAA stop codon.

Figure 7. (A) Schematic representation of the VCSM13-derived D3 helper phage genome deleted for the D1 and D2 domains of the g3 gene.

15   domains of the g3 gene. The D3 part of the g3 gene encodes the carboxy-terminal part of the g3 protein enabling the generation of stable, but essentially non-infectious helper phages. The D3 helper phage genome is identical to the CT helper phage genome. (B) Sequence of the D3 domain

20   of g3 present in the nucleic acid shown in (A). The BamHI site at position 3488 as well as the GTG start en TAA stop codon are underlined.

Figure 8. Codon usage adaptation in the g3 gene and the D3

25   domain to prevent homologous recombination during helper phage production and during phage display library amplification. The left panel shows the obtained one-letter coded amino acids and the right panel shows the optimal codons.

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Figure 9. Schematic representation of the pUC-g3 helper plasmid harboring the full length g3 gene under the control of the lac promoter and further harboring an ampicillin resistance gene (Amp R) and a ColE1 origin of

35   replication (ORI).

Figure 10. Schematic representation of the VCSM13-derived
N2CT helper phage genome deleted for the N1 (D1) domain
of the g3 gene that confers infectivity to the phage.

5   **Figure 11.** Schematic representation of the VCSM13-derived
p3-minus helper phage genome expressing the g3 leader
followed by only seven amino acids. At the DNA level, a
large part of the N2 (D2) domain and the entire CT (D3)
domain are still present The former C-terminal region of
10   gene III is present but does not encode functional protein
through frame shift and therefore includes many in-frame
stop codons.

SUMMARY OF THE INVENTION

The current invention provides methods and means that substantially lack the drawbacks outlined above and that

5   are characterized in providing novel phage particles such as chimaeric phages, novel helper phages, libraries comprising said chimaeric phages and methods and means to produce said chimaeric phages and said helper phages.

The invention provides a chimaeric phage having a

10   coat comprising a mixture of proteins, said mixture comprising a fusion protein wherein a proteinaceous molecule is fused to a functional form of a phage coat protein, said mixture further comprising a mutant form of said phage coat protein, said mutant form being impaired

15   in binding to a host cell receptor. The invention also provides a chimaeric phage having a coat comprising a mixture of proteins, said mixture comprising a fusion protein wherein a proteinaceous molecule is fused to a phage coat protein, or to a fragment or derivative

20   thereof, and wherein said fusion protein is functional so as to render the chimaeric phage infectious, said mixture further comprising a mutant form of said phage coat protein, said mutant form being impaired in binding to a host cell receptor.

25   In one embodiment, the invention provides a helper phage comprising nucleic acid encoding phage proteins or functional equivalents thereof that are essential for the assembly of said helper phage, said nucleic acid further encoding a mutant form of a phage coat protein, said

30   mutant form being impaired in binding to a host cell receptor, and wherein said helper phage does not comprise nucleic acid encoding a functional form of said phage coat protein.

The invention further provides methods and means for

35   producing phage particles, chimaeric phages, infectious phages and helper phage according to the invention. The

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invention also provides phage collections such as phage
display libraries comprising chimaeric phages and/or
infectious phages according to the invention.

DETAILED DESCRIPTION OF THE INVENTION

　　　　The present invention provides a chimaeric phage
having a coat comprising a mixture of proteins, said
5　mixture comprising a fusion protein wherein a
proteinaceous molecule is fused to a functional form of a
phage coat protein, said mixture further comprising a
mutant form of said phage coat protein, wherein said
mutant form is characterized in that a phage comprising no
10　wild type phage coat protein from which said mutant form
is derived and having a coat comprising said mutant form
and no copies of said functional form, is less infectious
than a phage comprising no wild type phage coat protein
from which said mutant form is derived and having a coat
15　comprising said mutant form and at least one copy of said
functional form. In one embodiment of the invention, said
mutant form is characterized in that a phage comprising no
wild type phage coat protein from which said mutant form
is derived and carrying said mutant form and no copies of
20　said fusion protein is less infectious than a phage
comprising no wild type phage coat protein from which said
mutant form is derived and carrying in addition to said
mutant form at least one copy of said fusion protein.
Preferably, said mutant form is characterized in that a
25　phage comprising no wild type phage coat protein from
which said mutant form is derived and carrying said mutant
form and no copies of said fusion protein or said
functional form is non-infectious. More preferably, said
mutant form is further characterized in that a phage
30　having a coat comprising said mutant form in the presence
or absence of copies of said functional form, is stable.
Stable as used herein means that the part of g3p that is
still present in said mutant form (and that is also
present in said functional form) ensures features such as
35　DNA binding and rigidity of the phage, but does not
contribute to infectiousness of said phage as do the

domains in said functional form that are not present in
said mutant form. The invention also provides an
infectious phage containing at least one copy of a mutant
form of a phage coat protein, wherein said mutant form has
5   lost the ability to mediate infection of a natural host by
said infectious phage.

In a preferred embodiment, said phage coat protein is
the g3 protein (g3p) present in the coat of phages such as
M13 and R408. More preferably, said mutant form comprises
10 a mutation in the D1 and/or the D2 region of g3p. In
another preferred aspect of the invention said chimaeric
phage and/or said infectious phage are part of a phage
collection such as a phage display library. In a more
preferred aspect of the invention such a phage collection
15 consists essentially of chimaeric phages or infectious
phages provided by the invention. Also preferred are
chimaeric phages or infectious phages according to the
invention that comprise binding moieties, such as
antibodies or fragments thereof as part of said fusion
20 protein.

In another embodiment the invention provides a method
for producing a phage particle comprising the steps of:
providing a host cell with a first nucleic acid encoding a
fusion protein, said fusion protein comprising a
25 proteinaceous molecule fused to a functional form of a
phage coat protein; providing said host cell with a second
nucleic acid encoding a mutant form of said phage coat
protein, said mutant form being characterized in that a
phage comprising no wild type phage coat protein from
30 which said mutant form is derived and having a coat
comprising said mutant form and no copies of said
functional form is less infectious than a phage comprising
no wild type phage coat protein from which said mutant
form is derived and having a coat comprising at least one
35 copy of said functional form, and wherein said host cell
comprises an additional nucleic acid sequence encoding at

least all other proteins or functional equivalents
thereof, that are essential for the assembly of said phage
particle in said host cell; and culturing said host cell
to allow assembly of said phage particle. The invention
provides a method for producing a phage particle, said
method comprising the steps of: providing a host cell with
a first nucleic acid encoding a fusion protein, said
fusion protein comprising a proteinaceous molecule fused
to a functional form of a phage coat protein; providing
said host cell with a second nucleic acid encoding a
mutant form of said phage coat protein, said mutant form
being impaired in binding to a host cell receptor; and
culturing said host cell to allow assembly of said phage
particle. Preferably, these methods according to the
invention for producing a phage particle are applied for
producing said chimaeric phage and/or said infectious
phage. More preferably said method is applied for
producing a phage particle such as a chimaeric phage or an
infectious phage provided by the invention that comprise
nucleic acid encoding said mutant form under the control
of a controllable promoter such as the AraC/BAD promoter,
the lac promoter or the psp promoter.

In another embodiment the invention provides a helper
phage comprising nucleic acid encoding phage proteins or
functional equivalents thereof that are essential for the
assembly of said helper phage, said nucleic acid further
encoding a mutant form of a phage coat protein, wherein
said mutant form is characterized in that a phage
comprising no wild type phage coat protein from which said
mutant form is derived and having a coat comprising said
mutant form and no copies of a functional form of said
phage coat protein, is less infectious than a phage
comprising no wild type phage coat protein from which said
mutant form is derived and having a coat comprising at
least one copy of said functional form, wherein said
functional form is characterized in that it renders a

phage particle carrying said functional form in its coat
infectious, and wherein said helper phage does not
comprise expressible nucleic acid encoding said functional
form. The invention provides a helper phage comprising
nucleic acid encoding phage proteins or functional
equivalents thereof that are essential for the assembly of
said helper phage, said nucleic acid further encoding a
mutant form of a phage coat protein, said mutant form
being impaired in binding to a host cell receptor, and
wherein said helper phage does not comprise nucleic acid
encoding a functional form of said phage coat protein.

In yet another embodiment the invention provides a
method for producing a helper phage comprising the steps
of: providing a host cell with a first nucleic acid
encoding a functional form of a phage coat protein;
providing said host cell with a second nucleic acid
encoding a mutant form of said phage coat protein, wherein
said mutant form is characterized in that a phage
comprising no wild type phage coat protein from which said
mutant form is derived and having a coat comprising said
mutant form, is less infectious than a phage comprising no
wild type phage coat protein from which said mutant form
is derived and having a coat comprising at least one copy
of said functional form, wherein said host cell comprises
an additional nucleic acid sequence encoding at least all
other proteins or functional equivalents thereof that are
essential for the assembly of said helper phage in said
host cell; and culturing said host cell to allow assembly
of said helper phage. The invention also provides a method
for producing a helper phage comprising the steps of:
providing a host cell with a first nucleic acid encoding a
functional form of a phage coat protein; providing said
host cell with a second nucleic acid encoding a mutant
form of a phage coat protein, said mutant form being
impaired in binding to a host cell receptor, wherein said
host cell comprises an additional nucleic acid sequence

encoding at least all other proteins or functional
equivalents thereof that are essential for the assembly of
said helper phage in said host cell; and culturing said
host cell to allow assembly of said helper phage.

5   In another aspect the invention provides methods and
means for producing a phage particle such as a chimaeric
phage, an infectious phage or a helper phage according to
the invention, wherein separate nucleic acids encoding
either (1) a functional form of said phage coat protein
10   alone or fused to a proteinaceous molecule or (2) a mutant
form of said phage coat protein, each comprise codons in
the overlapping regions between the protein encoding
parts, that essentially do not render a homologous
recombination event between said separate nucleic acids.
15   In a preferred aspect said separate nucleic acids each
comprises non-interfering origins of replication and
unique selection markers.

        In another embodiment the invention provides a method
for the enrichment of a first binding pair member in a
20   repertoire of first binding pair members selected from the
group consisting of an antibody, an antibody fragment, a
single chain Fv fragment, a Fab fragment, a variable
region, a CDR region, an immunoglobulin or a functional
part thereof, said first binding pair member being
25   specific for a second binding pair member, said method
comprising the steps of: contacting a phage collection
comprising chimaeric or infectious phages according to the
invention, with material comprising said second binding
pair member under conditions allowing specific binding;
30   removing non-specific binders; and recovering specific
binders, said specific binders comprising said first
binding pair member. Said material may comprise second
binding pair members such as purified proteins,
recombinant proteins and/or proteins present in or on
35   cells. In a preferred embodiment, the invention provides a
method for the enrichment of a first binding pair member

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that furthermore comprise the steps of: recovering from a
phage a DNA sequence encoding said first specific binding
pair member; sub-cloning said DNA sequence in a suitable
expression vector; expressing said DNA sequence in a
5   suitable host; and culturing said suitable host under
conditions whereby said first specific binding pair member
is produced. A suitable expression vector may be a plasmid
vector comprising an active promoter that regulates the
expression of said first specific binding pair member in
10 suitable hosts like eukaryotic cells such as yeast cells
or mammalian cells.

In another aspect, the invention provides a nucleic
acid molecule comprising a sequence encoding a mutant form
of a phage coat protein, said mutant form being
15 characterized in that a phage, comprising no wild type
phage coat protein from which said mutant form is derived
and having a coat comprising said mutant form and no
functional form of said phage coat protein, is less
infectious than a phage comprising no wild type phage coat
20 protein from which said mutant form is derived and having
a coat comprising said mutant form and at least one copy
of said functional form of said phage coat protein,
wherein said functional form is characterized in that it
renders a phage carrying said functional form in its coat
25 infectious. Said nucleic acid molecule may furthermore
comprise all relevant nucleic acid encoding proteins that
are required for assembly of a phage in a host cell.

The invention provides a chimaeric phage having a
30 coat comprising a mixture of proteins, said mixture
comprising a fusion protein wherein a proteinaceous
molecule is fused to a functional form of a phage coat
protein, said mixture further comprising a mutant form of
said phage coat protein, wherein said mutant form is
35 characterized in that a phage comprising no wild type
phage coat protein from which said mutant form is derived

and having a coat comprising a mutant form of said phage
coat protein and no copy or copies of said functional form
of said phage coat protein, is less infectious than a
phage comprising no wild type phage coat protein from

5   which said mutant form is derived and having a coat
comprising at least one functional form of said phage coat
protein. 'Functional form' as used herein refers to a
phage coat protein that contributes significantly to the
infectiousness of the particle to which it is attached.

10   The phage coat protein itself is not infectious, but the
functional form renders the phage particle to which it is
attached infectious. Besides the contribution to
infectivity of the phage particle, the phage coat protein
also sustains other functions such as stabilization of the

15   phage particle. A mutant form of a phage coat protein
according to the invention may render the phage particle
less infectious or non-infectious but should still sustain
other functions of said phage coat protein such as
stabilization of the phage. A phage that comprises no wild

20   type phage coat protein from which said mutant form is
derived and comprises no functional forms of said phage
coat protein but does contain only mutant forms of said
phage coat protein is less infectious than a phage that
comprises no wild type phage coat protein from which said

25   mutant form is derived and comprises one or more
functional forms of said phage coat protein next to the
mutant forms of said phage coat protein in its coat. 'Less
infectious' as used herein may also mean non-infectious.
Although a chimaeric phage of the invention comprises at

30   least one copy of the mutant form of said phage coat
protein in its coat, the chimaeric phage has infectious
capability because it also comprises at least one
functional form of said phage coat protein in its coat. A
'functional form of a phage coat protein' as used herein

35   also means a part, derivative and/or an analogue thereof
that still harbors functionality in rendering the phage

infectious to which it is attached. 'Mutant form of a
phage coat protein' as used herein also means a part, a
derivative and/or an analogue of said mutant form, wherein
said mutant form is characterized in that a phage,
5   comprising no wild type phage coat protein from which said
mutant form is derived and having a coat comprising only
mutant forms of said phage coat protein or parts,
derivatives and/or analogues thereof, is less- or non-
infectious as compared to a phage comprising no wild type
10 phage coat protein from which said mutant form is derived
and having a coat comprising at least one functional form
of said phage coat protein.

        In a preferred embodiment said phage coat protein is
the g3 protein (g3p) that in a wild type or functional
15 form renders a phage to which it is attached, infectious.
As is outlined in Figure 2 and 3, certain parts of the g3
protein are involved in the recognition of host cell
receptors. A mutant form is 'impaired' in receptor binding
if an alteration in the g3 protein renders the receptor to
20 be recognized and bound by the g3 protein, or parts
thereof, to a lower extend than if no alteration is
present. Therefore, 'impaired' as used herein means that
the coat protein, such as g3p, binds the host cell
receptor less efficiently, or that g3p has completely lost
25 the ability to bind and/or recognize the host cell
receptor. In a preferred embodiment the mutant form of
said phage coat protein comprises an alteration in the g3
protein consisting of a mutation in either the D1 region,
the D2 region or both. An 'alteration' or 'mutation' as
30 used herein means one or multiple point mutations,
stretches of mutations, deletions, substitutions,
replacements and/or swapping of parts. In a more preferred
embodiment said alteration in the g3 protein is a deletion
of substantially all of the D1 and/or the D2 region. Said
35 alteration may also mean a substitution of the deleted g3
protein part by a protein or a peptide not contributing to

the infectivity of the helper phage, the chimaeric phage,
the infectious phage or the phage particle.

In a more preferred embodiment of the invention a
chimaeric phage or an infectious phage according to the
invention comprises a nucleic acid encoding a fusion
protein, wherein a proteinaceous molecule is fused to a
functional form of said phage coat protein and wherein
said chimaeric phage of the invention comprises a M13,
M13K07, VCSM13 or a R408 strain or a mutant, derivative or
analogue strain derived from either one of these strains.
A proteinaceous molecule according to the invention is
fused to said functional form of said phage coat protein
and comprises a protein such as a ligand-binding moiety or
an immunoglobulin (such as an antibody). A proteinaceous
molecule may also mean a peptide such as a random stretch
of amino acids or a non-random stretch of amino acids such
as an antibody fragment or derivatives thereof (Fab
fragment, a single chain Fv fragment (scFv), a variable
region or a CDR region). A proteinaceous molecule can also
mean first specific binding pair member or can mean
fusions between different kinds of (fragments of) proteins
and/or fusions between (fragments of) proteins and (random
and non-random) peptides such as antibody-recognized tags.

A chimaeric phage of the invention relies
significantly for infection on the functional form of the
phage coat protein g3p and on the presence of part(s) of
said phage coat protein that contribute to the infectivity
of the phage. Said mutant form of said phage coat protein
is mutated in the part(s) of said phage coat protein that
render the phage infectious. The mutation is exemplified
in, but not limited to, deletions, residue- or fragment
substitutions, swaps and/or replacements by other protein
fragments rendering it less infectious. Said protein
fragments may or may not be related to phage coat proteins
or fragments thereof, and are essentially not capable of
inducing infection of the phage particle into a host cell.

In another embodiment the invention provides a phage
collection comprising a chimaeric phage or an infectious
phage according to the invention. Phages of the present
invention are particularly useful for the generation of
5   phage display libraries. Therefore, in a more preferred
embodiment said phage collection is a phage display
library. In an even more preferred embodiment said phage
collection consists essentially of chimaeric phages or of
infectious phages of the invention. A proteinaceous
10 molecule, such as (random or not random) stretches of
amino acids, peptides, protein parts or even entire
proteins can be fused to the phage coat proteins and can
form a first specific binding pair member. This fusion is
typically done at the terminal ends of the coat protein.
15 Addition typically does not affect the function of the
phage coat protein. Moreover, it also often does not
interfere with the function of the added moiety. Thus it
is possible to generate libraries that for instance can be
used to locate and to clone specific binding molecules.
20 Such libraries can comprise peptides or larger molecules.
Preferably, said larger molecules comprise a protein such
as an antibody or a functional part, derivative and/or
analogue thereof, such as full length heavy and/or light
chains from an immunoglobulin molecule, or fragments of
25 immunoglobulins such as Fab fragments, single chain Fv
(scFv) fragments, CDR regions, sole variable regions
and/or combinations of the above.

In another aspect the invention provides a method for
making a phage particle which comprises the steps of
30 providing a host cell with a first nucleic acid encoding a
fusion protein, said fusion protein comprising a
proteinaceous molecule fused to a functional form of a
phage coat protein or to a functional part, derivative
and/or analogue of said phage coat protein, providing said
35 host cell with a second nucleic acid encoding a mutant
form of said phage coat protein, said mutant form being

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characterized as described above and wherein less
infectious may also mean non-infectious, and culturing
said cell to allow assembly of said phage, said host cell
otherwise or additionally comprising nucleic acid encoding
5   at least all essential proteins, or functional equivalents
of said essential proteins for the assembly of said phage
particle. In a preferred embodiment the invention provides
a method wherein the nucleic acid encoding at least all
other proteins or functional equivalents thereof that are
10   essential for the assembly of said phage particle in said
host cell is comprised by a helper phage and said helper
phage is used to deliver the nucleic acid to said host
cell. In a more preferred embodiment the nucleic acid that
is delivered by said helper phage also comprises said
15   second nucleic acid encoding said mutant form of said
phage coat protein. In an even more preferred embodiment
said first and said second nucleic acid are separate
nucleic acids that each may comprise separate unique
selection markers to ensure that the host cell comprises
20   at least one copy of each separate nucleic acid and that
each comprise separate unique origins of replication to
ensure no interferences during replication. In another
aspect of the invention the number of possible homologous
recombination events between overlapping stretches of
25   nucleic acid sequences between the separate nucleic acids
is reduced due to the use of different codons within each
nucleic acid.

Using a method of the invention it is possible to
generate phage particles that comprise at least two
30   variants derived from the same coat protein. Such phage
particles have not been used in the art. The art teaches
the use of phage particles either having a coat holding
deleted g3 proteins, g3 proteins linked to heterologous
proteins, deleted g3 proteins linked to heterologous
35   proteins or limited numbers of g3 proteins, but no
mixtures thereof. The present invention provides novel

phage particles (chimaeric phages, infectious phages and
helper phages) in which the relative number of the g3p
variants (either fused to a heterologous moiety or not)
present in the coat can vary. Typically, one wants to
5   influence the relative amount of the various variants in
said phage coat. To that end it is preferred that
expression of said fusion protein and/or expression of
said mutant form of said phage coat protein is regulatable
(controllable). Preferably, this is achieved by regulating
10   the expression of the gene encoding said phage coat
protein at a transcriptional level. Thus, preferably,
expression of said fusion protein and/or said mutant form
of said phage coat protein is under the control of a
promoter that is well controlled. Examples of such
15   promoters are the lac promoter, the psp promoter and the
AraC/BAD promoter, the latter being influenced by the
concentration of glucose or arabinose in the medium.
Particularly advantageous is the AraC/BAD promoter. The
AraC/BAD promoter is preferred because it is a promoter
20   that is controlled in a very tight manner. This promoter
is for all practical purposes silent in the presence of
glucose and only slightly leaky in the absence of glucose.
This means that a low concentration or absence of glucose
renders the promoter active, resulting in up-regulation of
25   the gene of interest that is under the control of the
promoter. If, for example, the deletion mutant of g3
(lacking the D1 and D2 infectious regions) is under the
control of such a promoter, the relative number of
deletion mutants as compared to full length (or at least
30   functional) g3-fusion proteins would be low when the
glucose concentration is relatively high. In principle,
this system results in a tight regulation of the number of
deletion mutants and functional coat proteins such as g3
on the coat of a phage particle, and therefore the
35   percentage of such coat proteins and deletion mutants can
be regulated. In addition, the activity of the AraC/BAD

promoter can be regulated very tightly by the addition of
arabinose to the medium. The concentration arabinose used
determines the level of protein expressed in *E. coli* cells.
Therefore, optimal regulation of phage coat protein

5   content is accomplished by using this AraC/BAD promoter
and by altering the culturing conditions of the host cell.
The use of a promoter that is dependent on arabinose such
as the AraC/BAD promoter, instead of IPTG, such as the
lac-operon, prevents possible problems that will occur due

10 to co-encapsidation of the helper plasmid in viral
particles during helper phage synthesis. As described
above, it has been shown that co-encapsidation of plasmids
together with the phage genome does occur (Russel and
Model. 1989; Krebber et al. 1995; Rakonjac et al. 1997).

15 If co-encapsidation occurs with a lac driven helper
plasmid, it may compete with the lac driven vectors,
generally used for the phage display, resulting in the
production of infectious phage particles that will not
contain the g3p-X fusion product. This problem most likely

20 does not occur when the AraC/BAD promoter is used.
However, in the studies presented by the present invention
such competition problems with the lac promoter were not
observed. Therefore, other regulatable promoters such as
the psp and lac promoter can be used to generate phages

25 according to the invention. It is therefore also a part of
the invention to use such promoters as an alternative to
the AraC/BAD promoter.

A method of the invention for the production of a
phage particle is preferably used to produce a chimaeric

30 phage according to the invention. The host can be provided
with nucleic acid encoding a phage protein in any suitable
way. Preferably, however, said host is provided with a
helper phage according to the invention. A helper phage
according to the invention comprises nucleic acid encoding

35 other phage proteins or functional equivalents thereof
that are essential for the assembly of said helper phage,

said nucleic acid further encoding a mutant form of a
phage coat protein, wherein said mutant form is
characterized in that a phage comprising no wild type
phage coat protein from which said mutant form is derived

5   and having a coat comprising mutant forms of said phage
coat protein and no functional forms of said phage coat
protein, is less infectious than a phage comprising no
wild type phage coat protein from which said mutant form
is derived and having a coat comprising at least one

10   functional form of said phage coat protein and wherein
said helper phage does not comprise nucleic acid encoding
a functional form of said phage coat protein. With other
phage proteins is meant other than said functional form of
said phage coat protein and said mutant form of said phage

15   coat protein. Nucleic acid encoding the latter may be
provided to the host cell in an alternative fashion.
However, said helper phage may further comprise nucleic
acid encoding said functional form of said phage coat
protein or said mutant form or both. Preferably, said

20   helper phage does not comprise nucleic acid encoding said
fusion protein. In this way said helper phage is uniform
and may be used to produce phages that preferentially
comprise nucleic acid encoding said fusion protein in the
absence of nucleic acid encoding any other required helper

25   phage protein. Thus preferably said fusion protein and
said mutant form of said phage coat protein are encoded by
separate nucleic acids. Preferably, each of said separate
nucleic acids comprises a unique selection marker.
Preferably, said separate nucleic acids comprise non-

30   interfering origins of replication, wherein said origins
of replication do not compete with one another resulting
in bacterial cells that tolerate the separate nucleic
acids for the generation of new phage particles.

        The art teaches that it has been very difficult to

35   generate helper phage batches, wherein said helper phages
harbor nucleic acid that encodes all essential proteins

required for assembly of a phage particle in a bacterial
host cell and wherein said nucleic acid lacks a gene
encoding g3p. Subsequently, the art teaches that it is
difficult to produce phage libraries using such helper
5 phages. Several difficulties are known in the art that
hamper a proper generation of such helper phage batches.
The present invention provides methods and means and a
good combination of features such as the use of specific
origins of replication, selection markers and codons in
10 overlapping stretches of DNA, that enable the production
of phage batches containing high titers of useful helper
phages, that can subsequently be applied for the
generation of chimaeric or infectious phages according to
the invention. Therefore, in one embodiment the invention
15 provides methods and means for the production of helper
phages that carry functional forms and/or wild type forms
of a phage coat protein in their coat, but that
nevertheless lack nucleic acid encoding for said
functional form and/or said wild type form of said phage
20 coat protein. The invention provides a method for
producing a helper phage comprising the steps of:
providing a host cell with a first nucleic acid encoding a
functional form of a phage coat protein, providing said
host cell with a second nucleic acid encoding a mutant
25 form of said phage coat protein, wherein said mutant form
is characterized in that a phage comprising no wild type
phage coat protein from which said mutant form is derived
and having a coat comprising mutant forms of said phage
coat protein and no copies of said functional form, is
30 less infectious than a phage comprising no wild type phage
coat protein from which said mutant form is derived and
having a coat comprising at least one functional form of
said phage coat protein, and culturing said host cell to
allow assembly of said helper phage, said host cell
35 additionally comprising nucleic acid encoding at least all
other proteins or functional equivalents thereof that are

essential for the assembly of said helper phage in said
host cell. Preferably said method does not comprise the
incorporation of a wild type form of said phage coat
protein in said helper phage. Preferably said phage coat
5   protein is the g3 protein present in the coat of most, if
not all bacteriophages. Preferably, said other proteins
are encoded by said second nucleic acid, wherein
expression of said mutant form of said phage coat protein
and/or the expression of said functional form is regulated
10   by altering the culturing conditions of the host cell and
that is preferably under the control of a controllable
promoter such as the AraC/BAD, psp and lac promoter as
described above.

A phage coat protein is said to be contributing
15   significantly to the infectiousness of a phage when it
allows said phage, upon incorporation in a phage, to
recognize, bind and/or infect a bacterial host in a manner
comparable to a wild type version of said phage coat
protein. As used herein the terms less-infectious or non-
20   infectious refers to a phage, carrying no functional or
wild type forms of a phage coat protein, preferably g3p,
and that exhibits a significant impaired, diminished
infectious capability as compared to the wild type phage
(particle) as determinable in for instance plaque assays
25   well known to persons skilled in the art. As used herein,
the terms less- and non-infectious may also refer to a
decrease in host cell specificity. In general, a non-
infectious phage is unable to recognize, bind and/or enter
a bacterial cell as outlined for wild type phages in
30   Figure 2 and 3, whereas a less infectious phage is capable
of doing so with less efficiency. Preferably, a phage
having mutant forms infects the host cell with an
efficiency which is less than 50% compared to a phage
carrying at least a functional form, more preferably less
35   than 10%, still more preferably less than 1%, under
conditions which are otherwise comparable.

In embodiments wherein said phage coat protein comprises a g3p, said mutant form preferably comprises at least a structural part, derivative and/or analogue of the D3 region. Said structural part, derivative and/or

5 analogue comprise at least the functionality of g3p to allow assembly of stable phage particles.

A derivative of a protein as used herein comprises the same activity as the protein the derivative is derived from. When the protein is a functional form of a phage

10 coat protein the derivative comprises the same functionality. When the protein is a mutant form of a phage coat protein the derivative is also a mutant form of said phage coat protein. When the protein is a mutant form of a phage coat protein that renders a phage carrying only

15 mutant forms of said phage coat protein less infectious, the derivative also is a phage coat protein that renders a phage carrying only derivatives and/or mutant forms of said phage coat protein less infectious. Typical derivatives are proteins comprising one or more

20 conservative amino acid substitutions. However, derivatives may also comprise insertions and/or deletions. Furthermore, derivatives may also comprise swaps of amino acids or sequences of amino acids within the same protein or between two or more related and/or unrelated proteins.

25 An analogue of a protein as used herein comprises essentially the same activity as the protein the analogue is analogous to. When the protein is a functional form of a phage coat protein the analogue comprises the same functionality.

30   Prior to the invention it has been difficult to generate sufficient numbers of helper phages that lack a functional g3 gene or a part, derivative and/or analogue thereof in their genome, but that nevertheless are infectious for one round of infection through E.coli

35 cells. The present invention succeeds in generating such helper phages efficiently. Such helper phages carry

functional forms and/or wild type g3 proteins (g3p's) on
their surface, but nevertheless lack nucleic acid encoding
said functional form and/or said wild type g3 protein. The
invention further provides methods and means to generate
5   libraries comprising chimaeric phages with the help of
such helper phages. Preferably, phage display libraries
are generated. More preferably, libraries display a large
variety of single chain Fv (scFv) fragments. Using the
means and methods of the invention phage libraries can be
10   generated that contain a significant higher number of
infectious phages as compared to the number of non- or
less-infectious phages than were described and are present
in the art. At the same time the helper phages used to
produce such libraries become (through an infection round
15   in *E.coli* cells) essentially non-infectious, because the
g3 gene is not present in an infectivity-contributing form
in said phages. Thus after infection of a bacterial host,
said phages cannot spread to other bacterial cells except
of course through division of the already infected host
20   into daughter cells. Such libraries are therefore also
provided by the invention. The generated libraries are
particularly useful for panning experiments because the
titers of phages per milliliter are significantly higher
than was used in the art until the present invention.
25   Moreover, libraries of the invention display less a-
specific stickiness. Thus said libraries display less
false positives than libraries in the art. Moreover, after
one round of panning only phages that display a functional
form on their surface (preferably fused to a proteinaceous
30   molecule) can be amplified in *E.coli* cells, while phages
that do not carry any functional forms of said phage coat
protein but only carry mutant forms of said phage coat
protein are essentially non-infectious and cannot be
amplified on *E.coli* cells. Therefore, the number of
35   remaining phages that are used for a second round of
panning is significantly decreased. As a result of using

the chimaeric phages of the invention present in the
libraries provided by the present invention the number of
panning rounds is decreased and the number of relevant
binders is obtained in a much more sufficient manner as
5   was possible before the present invention.

          In another aspect the invention provides nucleic
acids and helper phages comprising said nucleic acids that
comprise genomic DNA sequences in which at least the
domains of g3p that are responsible and contributing to
10   infection are functionally removed. The invention also
provides helper phage genomic DNA's in which the leader
and at least the D3 domain are unaffected and fused
together. Said nucleic acids are preferably based on
VCSM13 and M13K07 genomic sequences. Due to a lack of a
15   functional D1 domain phage particles produced by said
nucleic acids are essentially non-infectious.
'Essentially' in this context means that no spread or at
least significantly less spread of said phages to other
bacterial cells than the production bacterium occurs
20   through g3p provided infectious features. This absence of
spread to other bacterial cells is due to the absence of a
functional form of g3p. If during production a source for
wild type form or functional form of g3p is provided,
produced phages can infect a bacterium. However, if said
25   bacterium produces phages as a result of the infection
then a resulting phage particle is not capable of
infecting another bacterium unless again a source for
infectious g3p is provided during production. A chimaeric
phage or an infectious phage of the invention preferably
30   comprises a part of g3p that ensures the generation of a
stable phage particle after one round of infection in
E.coli cells. To this end said helper phage preferably
comprises a nucleic acid encoding a mutant form of said
g3p. Preferably, said mutant form comprises D3 or a
35   functional part, derivative and/or analogue thereof.

A phage display library can for instance be generated
by providing a collection of bacteria with a library of
nucleic acids encoding g3p fused to a range of different
proteinaceous molecules and infecting said bacteria with
5   helper phages of the invention. In a particularly
preferred embodiment a library of phage display particles
that is produced with these helper phages contains phages
that do not carry any infectious parts of g3p on their
surface and phages that carry one or two full length g3p-X
10   fusions, next to non-infectious or less-infectious parts
of g3p deletion proteins. Phages in these library mixtures
that do not express g3p-X fusion proteins cannot infect
bacteria anymore, since they were generated in the absence
of infectious g3p parts that are not fused to X (X
15   comprises a proteinaceous molecule or a fusion partner of
interest such as immunoglobulins or fragments of
immunoglobulins such as Fab fragments or scFv fragments).

In one aspect the invention provides helper phages
that combine the presence of a selection marker with the
20   presence of a bacterial origin of replication (ORI) to
overcome the described problems in the production of g3-
minus helper phages and subsequently for the generation of
phage display libraries. The presence of such a
combination ensures the production of large amounts of
25   helper phages and/or helper genomes. g3-minus helper
phages with an ORI and a resistance marker can be made
from the helper phages VCSM13 and M13K07. These helper
phages, unlike M13 or R408, do contain a kanamycin
resistance gene from the Tn903 transposon and a P15A ORI
30   that are both inserted in the intergenic region of the
phage genome. Another aspect of the invention is the fact
that because of this resistance gene and the presence of
this particular ORI, these helper phages can grow easily
in large quantities, while empty, or no plasmid- or no
35   genome-containing bacteria are removed under the selection
pressure, and that no interferences occur between ORI's

from the phage genome and the helper plasmid or between
ORI's from the phage genome and the display library
plasmids, when both nucleic acids are present in the same
bacterial host cell. VCSM13 and M13K07 contain the P15A

5   ORI. To prevent the disappearance of the helper genome or
the helper vector the ORI's should not cause any
interferences and therefore P15A derived ORI's are not
used in the vector. For the vector applied for the
production of helper phages, the ColE1 ORI was chosen.

10 Besides the features and effects mentioned above, it is
also important that the helper vector for the generation
of helper phages does not carry an F1 ORI to prevent the
incorporation of the helper vector in the phage particle
instead of the viral genome.

15   The invention also provides vectors enabling a
regulated expression of the mutant form and/or the
functional form of g3p by the use of a regulatable
promoter and that furthermore contain a resistance gene
that is different from the kanamycin resistance gene

20 present in the helper genome. This complementary
resistance is here provided by the beta-lactamase
(ampicillin) gene since its product is relatively stable
and ensures complete killing of bacteria that do not
express the gene product.

25   The pBAD/gIII vector (Invitrogen) can be used as a
backbone vector for the production of helper vectors of
the invention. Alternatively, vectors such as pUC19 can be
used. Preferably, further features of these basic helper
vectors are that regions of sequence homology are

30 minimized which significantly decreases the possibility of
homologous recombination.

The invention further provides the use of TOP10, LMG
and/or XL1blue bacterial host cells for the production of
helper phages that contain a g3-minus genome but are

35 nevertheless infectious due to the g3p present on the
phages because it was delivered by the helper vector. The

genotype of the TOP10 and LMG bacteria ensures that they
can transport arabinose into the cell but that they cannot
metabolize it (genotype: araABCD- and araEFGH+). In
addition, the TOP10 bacteria are recA and endA deficient

5   which diminishes the chance of recombination and mutation.
Furthermore, the TOP10 bacteria are F-, which makes them
resistant to phages that might contaminate phage batches
of interest.

        The present invention also provides the sequence of

10   the VCSM13 phage genome and use thereof for the purpose of
phage display library construction, cloning phage and
plasmid mutants useful in general molecular biology
methods and means.

        The present invention provides further a partially

15   deleted g3 gene that is still present in the helper phage
genome to provide stable but essentially non-infectious
helper phages that harbor infectious g3p's on their coat.
The invention describes this partially deleted g3 gene,
that is made synthetically by using synthetic primers in

20   such a way that the functionally deleted g3 gene encodes
the same protein on an amino acid level as compared to the
other part of the g3 gene that is present in the same
bacterium, but the codons that are used do not lead to
homologous recombination events. Because the leader

25   sequences in the different settings are very different
there is no need to change these regions in the helper
genome, phage display vector (with the scFv encoding
genes) or helper vector. In principle it does not matter
whether the g3 gene in the helper genome, in the phage

30   display vector or helper vector has been changed as long
as the two overlapping (in amino acid content) and
previously homologous g3 parts that are introduced into
one *E.coli* cell do not match.

        The present invention describes the use of codon

35   changes in the g3 gene for the production of helper phages
that are infectious due to g3p's encoded by the helper

vectors but that lack a wild type or at least an
infectious g3 gene in their genome and for the production
of chimaeric phages according to the invention. The use of
codon changes ensures a diminished chance for homologous

5   recombination effects that might occur during the process
of helper phage generation. The invention preferably
provides the use of codon changes in the g3 gene or parts
thereof for the generation of phage display libraries in
which the helper phage genome, that is brought into an

10   *E.coli* cell together with nucleic acids encoding for g3p-X
fusion proteins, is not homologous to the g3 gene present
in the DNA encoding for the g3p-X fusion protein. These
codon changes ensure that the chance for homologous
recombination events in the generation of phage display

15   libraries is significantly decreased through which the
quality of these libraries and uses thereof are
significantly improved.

20

EXPERIMENTAL PROCEDURES

Primers

        The following primers (obtained from Genset or
5   Invitrogen) were used in the generation of the different
    vectors and helper phage genomic constructs. Most
    restriction enzymes hardly or fail to digest DNA if their
    corresponding palindrome is near the end of the DNA.
    Therefore a stretch of 8 nucleotides was added to the 5'
10 end of the D3, g3-minus and g3 ORF primers in which this
    stretch is an A/T rich non-hybridising 8-mer.

D3 primers

D3 BamHI Forward

15 5'-GGATCCTCTGGTTCCGGTGATTTTGATTATG-3'

D3 BamHI Backward

5'-GGATCCAGCGGAGTGAGAATAGAAAGGAAC-3'

g3-minus primers

20 **g3 minus HindIII Forward**

5'AAGCTTCTGCGTAATAAGGAGTCTTAATCATGC-3'

g3 minus HindIII Backward

5'-AAGCTTGTTGAAAATCTCCAAAAAAAAAGGC-3'

25 g3 ORF primers

g3 ORF NcoI Forward

5'-CCATGGCTGAAACTGTTGAAAGTTGTTTAGC-3'

g3 ORF XbaI Backward

5'-TCTAGATTAAGACTCCTTATTACGCAGTATG-3'

30

CT and N2CT primers

SnaBIclon

5'-TTAGGTTGGTGCCTTCGTAG-3'

Bamlead

5'-GGATCCAGCGGAGTGAGAATAGAAAGG-3'

BglN2

5'-AGATCTGGTACTAAACCTCCTGAGTACGG-3'

BamCT

5'-GGATCCTCTGGTTCCGGTGATTTTGATTATG-3'

PacIclon

5'-TTGCTTCTGTAAATCGTCGC-3'

pUC-g3 primers

H3leadA

5'-CAAATTCTATTTCAAGGAGACAGTCATAATGAAAAAATTATTATTC
GCAATTCCTTTAG-3'

H3leadB

5'-GATTACGCCAAGCTTGCATGCAAATTCTATTTCAAGGAGA-3'

p3endEco

5'-GCTAACATACTGCGTAATAAGGAGTCTTAAGAATTCCAGTTCTTT-3'

PCR reactions and product isolation

PCR reactions with D3, g3-minus and g3 ORF primers
were as a standard (except for the elongation time of the
DNA synthesis cycle step) performed using the following 50
µl hot start PCR scheme and the AmpliTaq PCR kit from
Perkin Elmer: 1 µl 10mM dNTP (Roche Diagnostics), 4 µl
25mM MgCl$_2$, 5 µl 10X PCR buffer supplied with the kit, 5
µl 2.5 µM Forward primer, 5 µl 2.5µM Backward primer, 0.3
µl 5 units/µl AmpliTaq, 10-50 ng template, sterile
bidestilled water. All components were kept on ice until
placing in the pre-heated PCR block. The standard program
was as follows. 12 cycles of 25 sec 94°C, 52°C annealing
for 25 sec, 72°C polymerisation ending with one cycle of
72°C for 7 min followed by storage at 4°C. The time of
polymerisation for new helper genome synthesis was 12 min,
and for g3 amplification and for AraC gene and AraC/BAD
promoter amplification this was set at 90 sec. PCR

reactions with pUC-g3 primers were performed as above, but
with Pwo polymerase (Boehringer Mannheim) instead of
AmpliTaq, and with the following program: 30 cycles of 45
sec 94°C, 50°C annealing for 30 sec and 72°C
5   polymerisation for 1 min, ending with one cycle of 68°C
for 8 min followed by storage at 4°C. PCR reactions with
CT and N2CT primers were performed as above, but with Taq
polymerase (Gibco) instead of AmpliTaq, and with the
following program: 25 cycles of 30 sec 96°C, 53°C
10   annealing for 30 sec and 72°C polymerisation for 2 min,
ending with one cycle of 72°C for 10 min followed by
storage at 4°C. An exception was the PCR reaction with
N2CT primers BglN2 and PacIclon, which was performed using
Taq DNA polymerase recombinant (Invitrogen) with the
15   following program: 30 cycles of 45 sec 94°C, 55°C
annealing for 30 sec and 72°C polymerisation for 2.5 min,
ending with one cycle of 72°C for 10 min followed by
storage at 4°C.
      All PCR products were separated on 0.5%-1% TBE
20   agarose gels containing 100 ng/ml ethidium bromide. After
imaging the desired fragments were cut out using sterile
disposable chirurgical knives and isolated with Qiagen's
gel purification kit according to the guided protocol.

25   Ligation reactions
      Ligation reactions were as a standard performed in
the following reaction mixtures:
      50 ng vector or helper genome
      25 ng insert
30   4 µl 5X ligation buffer (Gibco BRL)
      1 µl T4-ligase (Gibco-BRL, 200 units/µl)
      sterile bidestilled water to 20 µl
However, for the construction of the pUC-g3 helper
plasmid, T4 DNA ligase (Roche, 1 unit/µl) was used, while
35   T4 DNA ligase (NEB, 400 units/µl) was used in the
construction of the helper phage genomes CT, N2CT and p3-

minus. The mixtures were incubated overnight at 6-16°C.
Then, 30 µl sterile water, 5 µl K-acetate 3M pH 4.8 acidic
acid adjusted (KAc), 1 µl glycogen 10 mg/ml and 50 µl
isopropanol or 96% ethanol were added and mixed

5   thoroughly. After 15 min of precipitation the tubes were
centrifuged at maximum speed at 4°C for 10 min. The pellet
was once washed with 1 ml 70% ethanol and after drying
dissolved in 10 µl sterile water. Half of this volume was
used for electroporation together with 50 µl competent

10 cells.

Sequencing
        Sequencing of the clones was performed according to
the instruction guide sent along with the ABI PRISM BigDye

15 Terminator Cycle Sequencing Kit (Applied Biosystems) at
50°C annealing temperature. All clones were sequenced in
order to verify the correctness of the products. The
sequence of the VCSM13 helper phage genome was determined
by the primer-walking method on both strands, a method

20 generally known to persons skilled in the art.

Electroporation
        All bacterial strains, except those that were used
for the helper phage production, were acquired from

25 manufacturers as electroporation competent cells with the
highest competence available and transformed according to
the manufactures protocol using 0.1 cm cuvettes (BioRad).
The production of helper phages, however, is dependent on
TOP10 or LMG cells (Stratagene) containing the helper

30 plasmid (pBAD/gIII-g3). These cells were made competent
and stored at -80°C until use as follows: One colony of
the bacteria was used to inoculate in 10 ml 2X TY with
ampicillin (100 µg/ml) and for LMG also with tetracycline
(10 µg/ml) and cultured by vigorously shaking at 30°C

35 overnight. Next, the cultures were spun down at 3000 rpm
for 5 min. The pellet was resuspended in 500 ml fresh 2X

YT including the antibiotics and cultured until OD 0.5 in
a 2 l Erlenmeyer flask on a shaking platform at 37°C.
These cells were allowed to cool on ice water for 45 min
and centrifuged in pre-cooled buckets and rotor at 3000

5   rpm at 4°C for 25 min in a Sorvall centrifuge using a GLA-
3000 rotor. The supernatant was discarded and the cells
were slowly and carefully resuspended in 100 ml ice-cold
10% glycerol. The centrifugation and glycerol steps were
repeated twice. The final pellet was taken up very

10 carefully in 5 ml 10% ice-cold glycerol and aliquoted in
pre-cooled eppendorf tubes. Next, these tubes were
immersed in a mixture of ethanol and dry ice for 5 min to
ensure very quick freezing of the cells. The tubes
containing the electrocompetent cells were stored at -80°C

15 until use. A comparable procedure was used to make
competent cells of XL-1 (Stratagene) containing the helper
plasmid pUC-g3.

Phage production

20   The desired F^+ E.coli strain is inoculated in 2X YT
medium containing the required antibiotics and cultured at
37°C at 220 rpm until OD 0.2. The (helper-) phage is added
to the culture and incubated for 30-45 min at 37°C in a
non-shaking waterbath. Then, kanamycine (50 µg/ml) is

25 added to the cells and cells are further incubated at 220
rpm at 37°C for 30-45 min. Subsequently, this solution is
spun at 3500 rpm at room temperature for 15 min. The
supernatant is removed carefully and the pellet is brought
to the desired volume of 2X YT medium containing all

30 required antibiotics. Cells are cultured overnight at 30°C
for a phage display library and at 37°C for regular
(helper-) phages on a shaking platform.

Titer determination

35   One colony of Xllblue (Stratagene) is inoculated in 5
ml 2X YT containing 10 µg tetracycline per ml (YT-T) in a

50 ml tube (Falcon) and cultured at 37°C at 220 rpm
overnight. 200 μl of this culture is added to 5 ml YT-T
and cultured until OD 0.2. Then, the phage stock is
diluted in YT and a dilution series as required is made to
5   determine the number of plaque forming units. For each
dilution step 100 μl of the O.D. 0.2 XL1Blue culture is
taken and added to 100 μl of the phages. This mixture is
incubated for 25 min at 37°C in a water bath (not
shaking). The 200 μl of bacterial cells is pipetted onto a
10   2X YT-broth plate containing the required antibiotics. The
suspension is spread using a sterile glass rod. After
drying the plates they are inverted and transferred into a
37°C incubator. After overnight culture the number of
colonies are counted. Each colony indicates the presence
15   of 1 infective phage particle in the original phage
solution. The number of infectious particles per ml of the
analyzed stock is calculated. The phage particles are
ELISA tested according to the protocol supplied with anti-
M13 and anti-M13-HRP conjugate (Pharmacia).
20

Isolation of DNA from phages
      An overnight phage culture is grown as described
above. If a large scale isolation of DNA was required the
BioRad Plasmid Maxi Prep kit was used according to the
25   manufactures instructions, except for the elution step
which is done with 10 mM Tris pH8.5 at 65°C for 10 min.
Small or medium scale isolations were performed using
Qiagen's mini-prep kit according to the instruction
supplied with the kit except for the elution step. The
30   elution step here was performed at 65°C for 10 min.

PEG precipitation
      The medium containing bacteria and phages is
collected in 450 ml buckets. The mixture is spun in a pre-
35   cooled Sorvall centrifuge using a GSA-3000 rotor at 8000
rpm for 20 min. Then, 90 ml 20% PEG/2.5 M NaCl is pipetted

into clean 450 ml buckets. 360 ml of the supernatant of
the centrifuged medium containing the phages is brought
into the PEG containing buckets and mixed well. The
mixture is set on ice water for 2 h or overnight in the
5   fridge. The precipitate is pelleted by centrifugation in a
pre-cooled Sorvall centrifuge at 8000 rpm for 20 min. The
supernatant is decanted and the buckets are left to drip
out for 5 min in order to remove as much Precipitation
buffer as possible. Subsequently, 32 ml PBS/1% bovine
10   serum albumin (BSA) is added to the 450 ml buckets
containing the pelleted phages and buckets are rotated on
a bottle roller for 15 min. The solution is transferred to
a SS-34 compatible centrifuge tube and spun in a pre-
cooled Sorvall centrifuge containing a SS-34 rotor (or
15   equivalent equipment) for 25 min at 13,000 rpm. This step
removes all kind of debris and small bacteria. In the
meantime, the plunger is removed from a 50 ml syringe and
attached to a 0.45 µM filter (Whatmann). The centrifuged
supernatant is transferred into the syringe and pushed
20   through the filter. This step removes all small bacteria
and other cells. 8 ml 20% PEG/2.5 M NaCl is added and
mixed well. The tubes are set on ice for 1 h. The high-
speed centrifugation step is repeated as described above.
The supernatant is decanted and the tube is let to drip
25   out on a paper towel for 5 min. The phage pellet is
dissolved in 5 ml PBS/1% BSA. Then, 5 ml 100% glycerol is
added to the phage solution and mixed well. Phages are
stored at -20°C. Typically, the solution contains
approximately 2 to 5×10^{13} infectious phage particles per
30   ml.

EXAMPLES

To illustrate the invention, the following examples are provided, not intended to limit the scope of the invention.

Example 1.

Cloning of the pBAD/gIII-g3 helper vector.

The full Open Reading Frame (ORF) of the g3 gene was generated by using M13KO7 (Gibco-BRL) DNA as a template in a standard PCR reaction together with g3 ORF NcoI Forward and g3 ORF XbaI Backward primers. The purified PCR product and the pBAD/gIII vector were both digested with NcoI (NEB) and XbaI (Roche Diagnostics) simultaneously in buffer H (Roche Diagnostics) for 4 h at 37°C. After ligation, isolation, electroporation in TOP10 (Stratagene) and LMG (Stratagene) cells, two correct clones were selected by sequencing and grown on a large scale followed by the isolation of the DNA. The DNA was reprecipitated with 70% ethanol and in the presence of KAc and the pellet was washed twice with 70% ethanol. After drying the DNA was dissolved in sterile bi-destilled water and stored at -20°C until use. The resulting plasmid pBAD/gIII-g3 is depicted in Figure 5. This helper vector contains the full sized g3 gene under the control of the AraC/BAD promoter, ampicillin resistance gene and a ColE1 ORI as most important features.

Example 2.

Cloning of the g3-minus helper phage genome.

VCSM13 (Stratagene) is a widely used helper phage, however, its sequence is not publicly available. The full DNA sequence of the VCSM13 genome was determined and is depicted schematically in Figures 6A and in nucleotide order in Figure 6B. The use of g3 minus HindIII Forward and g3 minus HindIII Backward primers and M13KO7 and VCSM13 as

48

templates in a standard PCR reaction resulted in the
formation of a PCR product that contained HindIII sites at
both ends of the DNA. After separation, gel isolation and
purification, digestion with HindIII (Roche Diagnostics)

5   and re-purification of the DNA, the product was self-
ligated under standard ligation conditions and the
resulting helper phage genome, named g3-minus, was
electroporated into XL1Blue cells (Stratagene). The
transformed cells were resuspended in 5 ml 2TY medium and

10 cultured shaking at 37.°C for 1 h. Kanamycin was added to
an end-concentration of 50 µg/ml and the cells were
allowed to grow at the same conditions for another 5 h.
The culture was centrifuged at 3000 rpm for 15 min and the
supernatant passed through a 0.22 µM filter to remove

15 bacteria. At the same time, a culture of exponentially
growing XL-1 Blue bacteria was prepared. Fractions of the
filtrate (50-1000 µl), containing phage particles, were
added to 5 ml of XL-1 Blue bacteria and incubated at 37°C
for 30 min without shaking. The culture was centrifuged

20 again, the supernatant discarded and the cells were plated
on 2X YT-K-T plates and transferred into an incubator at
37°C for overnight growth. Eight correct clones that lack
the g3 ORF (checked through the BamHI site) and include
the introduced HindIII site were isolated and used for g3-

25 less helper phage production in the presence of the
pBAD/gIII-g3 helper plasmid. Only two clones that were
able to form phages in the presence of the helper plasmid
were kept. From these clones a large quantity of DNA was
isolated and stored for further experiments. The obtained

30 VCSM13-derived g3-minus helper phage genome, in which the
entire g3 gene except for the last six codons was replaced
by a HindIII site, is depicted schematically in Figure 6C,
while the g3-minus sequence is depicted in Figure 6D.

35

Example 3.

Cloning of the D3 helper phage genome with a partially deleted g3 gene.

The construction of helper phage genome that express
5   only the D3 part of the g3 gene was comparable to the
above described g3-minus helper phages with the exception
that the primers used were D3 BamHI Forward and D3 BamHI
Backward primers in order to generate the new genome. All
other procedures were the same as for the g3-minus
10   procedure, except for the use of BamHI instead of HindIII.
In the end, the DNA of two correct clones was kept and
stored at -20°C. The final VCSM13-derived construct of the
helper phage genome (named D3: it only expresses this part
of the g3 gene that does not contribute to the
15   infectiousness of the phage particle) is depicted in
Figure 7A, while the D3 sequence is depicted in Figure 7B.

Example 4.

Production of infectious helper phages that do not carry a
20   **gene encoding the wild type g3p.**

The procedures for generating g3-less and generating
partially deleted g3 (or D3 expressing) helper phages are
identical. Frozen competent TOP10 or LMG cells that
contain the pBAD/gIII-g3 helper plasmid were
25   electroporated using 100 ng helper phage DNA. After
recovery the cells were transferred into 4X 250 ml 2X YT-
K-A medium supplemented with 0.05% arabinose (Sigma).
Phages were produced during overnight culture at 37°C and
vigorously shaking. The next day the phages were purified
30   and stored according to the standard procedures of
precipitation and storage that were described supra. The
number of infectious particles and the number of phages
were determined by titration and ELISA procedures that
were also described supra. For g3-less helper phages
35   approximately $5x10^{11}$ infectious particles were synthesized

while for D3 approximately 5×10^{13} infectious phages were
formed using these procedures.

Example 5.

5 **Amplification and harvesting of phage display libraries**
containing infectious phages carrying g3p-scFv fusions and
non-infectious helper phages, using g3 minus and partially
g3 deleted helper phages.

A frozen library was inoculated as follows. In
10 general approximately 5-10 µl concentrated stocks were
inoculated in 25 ml 2X YT containing the required
antibiotics and 5% glucose and grown at 37°C with
vigorously shaking. At OD 0.3-0.4 (after about 2-3 h), a
500-1000 fold excess of helper phage was added. The medium
15 containing the helper phages and bacteria was incubated in
a water bath at 37°C without shaking for 25 min. Removal
of dead cells and excess of phage particles was realized
after a centrifugation step at 3000 rpm for 15 min. The
pellet was resuspended in 250 ml 2X YT with antibiotics
20 but without glucose and grown at 30°C with good aeration
overnight. The next day the formed phages were isolated
and stored using the standard PEG/NaCl procedure.

Example 6.

25 **Codon usage in g3 and the partially deleted g3 gene (D3)**
to prevent homologous recombination during helper phage
production and library amplification.

In order to prevent possible recombinations between a
genomic nucleic acid encoding the helper phage g3 protein
30 region (or a part thereof such as the D3 domain) and other
nucleic acids (like the phage display vector and the
AraC/BAD helper vector), a series of helper phages are
designed that contain changed codons within the g3p
region. Newly translated g3p's are identical to the wild
35 type g3 protein or protein part (D3). Due to these

changes, g3-ORF coding DNA domains cannot or barely
recombine with the phage display vectors or the AraC/BAD-
g3 helper vector. The codons that are used to generate
non-homologous g3 genes are depicted in Figure 8 and are
5   optimal for the E.coli transcription machinery.
PCR generation of helper phage genomes (VCSM13, M13K07,
D3, g3-minus or AraC/BAD) with g3-leader backward and g3
end forward primers with NotI restriction sites ensure
the generation of PCR products containing all helper
10   phage components and genes, except for the g3 ORF.
New g3 regions are constructed with overlapping primers
and are inserted in helper phages. The PCR-generated g3p
or parts thereof are digested with NotI and ligated in
the NotI digested PCR generated helper phage genome.
15   After transformation and selection of correct helper
genomes (with a new g3 gene), helper phages are grown as
described.

Example 7.

20   **Selection of thyroglobulin-interacting phages using a**
library amplified with helper phages comprising only the
D3 part of g3p in their genome.
         In order to validate the D3 helper phages in
standard phage selections, a selection was performed
25   using an antibody phage display library that was
amplified using the D3 helper phages as described above.
Procedures that were used were essentially as described
by De Kruif et al. (1995a). Briefly, thyroglobulin was
coated to a plastic tube. The tube was blocked in PBS
30   containing 2% milk (MPBS) whereafter the antibody phage
display library, also blocked in MPBS, was added to the
tube. The phages were allowed to bind for 2 h, whereafter
non-binding phages were removed by washing the tube in
PBS containing 0.1% Tween-20 as described by De Kruif et
35   al. (1995a). The binding phages were eluted in 50 mM
Glycin/HCl pH 2.2 (10 min at RT) and used to infect

freshly grown XL-1 Blue bacteria. The bacteria were
plated on 2TY agar plates containing the appropriate
antibiotics and glucose, incubated overnight at 37°C and
used to prepare an enriched phage display library; phage
5   D3 was again used as helper phage. The procedure was
repeated once, whereafter individual *E.coli* colonies were
used to prepare monoclonal phage antibodies. These
monoclonal phage antibodies were tested in ELISA for
their ability to bind specifically to the thyroglobulin
10   antigen. Results show that after two rounds of selection
25/46 colonies show positive binding to thyroglobulin.
Previously we found that by using a general VCS-M13 as a
helper phage, at least 3 rounds of selection were
required to obtain specific binders in this selection
15   format.

Example 8.
Selection of phages interacting with myeloma cells using
a library amplified with helper phages comprising only
20   **the D3 part of g3p in their genome.**
      In order to validate the D3 helper phages in
selections on intact cells, a selection was performed
using an antibody phage display library that was
amplified using the D3 helper phage. Procedures that were
25   used were essentially as described by De Kruif et al.
(1995a and 1995b). Briefly, myeloma cells (AML, CD33+,
CD34+) were obtained from the blood of a patient
undergoing treatment at the Utrecht University Hospital
(The Netherlands). 0.5 ml phage library was added to 3 ml
30   RPMI medium containing 10% FCS (RPMIS) and incubated on
ice for 15 min. The myeloma cells were added and the cell
suspension was rotated at 4°C for 2 h. The cells were
washed 5 times in 50 ml ice-cold RPMIS whereafter the
binding phages were eluted (in 50 mM Glycin/HCl pH 2.2
35   for 10 min at RT) and used to infect freshly grown XL-1
Blue bacteria. The bacteria were plated on 2TY agar

plates containing the appropriate antibiotics and
glucose, incubated overnight at 37°C and used to prepare
an enriched phage display library. Again, the D3
expressing helper phages were used as helper. The

5   procedure was repeated once, whereafter individual *E.coli*
colonies were used to prepare monoclonal phage
antibodies. These monoclonal phage antibodies were tested
in FACS procedures for their ability to bind myeloma
cells. Results show that 23 out of 41 clones tested bound

10 specifically to epitopes expressed on the myeloma cells.
Generally, three or more rounds of selection are required
to obtain similar numbers of binding phages using
identical procedures, with the exception of using VCS-M13
helper phages instead of the helper phages described by

15 the invention.

Example 9.

Cloning of the pUC-g3 helper plasmid.

As an alternative to the pBAD/gIII-g3 helper plasmid

20 described above, a pUC19-based helper plasmid for the
expression of wild-type g3p under the control of a lac
promotor was constructed. The use of H3leadA and p3endEco
primers and VCSM13 as a template in a PCR reaction
detailed above resulted in the formation of a PCR product

25 containing the g3 gene sequence with 28 additional
nucleotides upstream of the gene and 15 additional
nucleotides including an introduced EcoRI site
downstream. The use of H3leadB and p3endEco primers and
this PCR product as a template in a PCR reaction detailed

30 above resulted in the formation of an extended PCR
product containing the g3 gene sequence, with introduced
HindIII and EcoRI sites upstream and downstream of the
gene, respectively. The extended PCR product was cloned
into plasmid pCR4—BluntII-TOPO using the Zero Blunt TOPO

35 PCR Cloning Kit (Invitrogen) and the sequence of the
insert was checked. Plasmid pUC19 (New England Biolabs)

and the pCR4-BluntII-TOPO derivative containing the g3
gene were both digested with HindIII and EcoRI. The
fragment containing the g3 gene from the pCR4-TOPO
derivative and the vector fragment of pUC19 were purified
5   from gel and ligated. The resulting plasmid, named pUC-
g3, was electroporated into XL-1 cells (Stratagene) and a
correct clone was selected by sequencing DNA isolated
from the transformed cells. Helper vector pUC-g3, which
contains the g3 gene under the control of the lac
10 promotor, is depicted schematically in Figure 9.

Example 10.
Alternative cloning of helper phage genomes with a
partially deleted g3 gene.
15   In the cloning procedures described in example 2 and
3, a very large region of the phage genome is amplified
by PCR, with the risk of introducing artificial mutations
due to the limited fidelity of DNA polymerase. An
alternative strategy was applied to produce helper
20 genomes with a partially deleted g3 gene. In this
strategy, a part of the g3 sequence encoding the g3
leader, and a part of the g3 sequence encoding a C-
terminal portion of the g3 protein, were amplified
separately. The two fragments were combined in a shuttle
25 vector and subsequently cloned into the phage genome. To
distinguish the resulting constructs from the D3 helper
phage described in example 3, the generally accepted
alternative nomenclature for the g3 domains was used, in
which D1, D2 and D3 are named N1, N2 and CT,
30 respectively. The use of SnaBIclon and Bamlead primers
and VCSM13 as a template in a PCR reaction detailed above
resulted in the formation of a PCR product containing the
g3 leader sequence, with a native SnaBI site upstream and
an introduced BamHI site downstream. The use of BamCT and
35 PacIclon primers and VCSM13 as a template in a PCR
reaction detailed above resulted in the formation of a

PCR product containing the g3 CT domain, with an
introduced BamHI site upstream and a native PacI site
downstream. The use of BglN2 and PacIclon primers and
VCSM13 as a template in a PCR reaction detailed above

5   resulted in the formation of a PCR product containing the
g3 N2 and CT domains, with an introduced BglII site
upstream and a native PacI site downstream. All PCR
products were cloned into plasmid pCR4-TOPO using the
TOPO TA Cloning Kit for Sequencing (Invitrogen) and the

10   sequences of the inserts were checked. The pCR4-TOPO
derivative containing the leader sequence was digested
with NotI and BamHI, and the insert was isolated from
agarose gel. The pCR4-TOPO derivative containing the CT
domain was also digested with NotI and BamHI, and the

15   vector fragment was isolated. The insert was ligated with
the vector fragment, resulting in a pCR4-TOPO derivative
containing the g3 gene in which the N1 and N2 domains are
deleted. This plasmid and VCSM13 were both digested with
SnaBI and PacI. After isolation the plasmid insert was

20   ligated with the VCSM13 vector fragment. The sequence of
the insert in the resulting phagemid, named CT and
identical to D3 depicted in Figure 7A, was checked. A
comparable procedure was used to construct a phagemid
lacking only the N1 domain. For this construct, the pCR4-

25   TOPO derivative containing the N2 and CT domains was
digested with NotI and BglII, and the vector fragment was
isolated. The insert containing the leader sequence was
ligated with the vector fragment (the cohesive ends of
BglII and BamHI are compatible), resulting in a pCR4-TOPO

30   derivative containing the g3 gene in which the N1 domain
is deleted. This gene was sub-cloned to VCSM13 as
described above and the sequence of the insert in the
resulting phagemid, named N2CT, was checked. The VCSM13-
derived helper phage genome N2CT is depicted

35   schematically in Figure 10.

Example 11.

Cloning of the p3-minus helper phage genome.

In the cloning of the g3-minus helper phage genome described in example 2, almost the entire g3 sequence was deleted. The g3 gene overlaps the ribosome-binding site of the g6 gene and may contain other important features as well. An alternative cloning strategy was applied, in which part of the N2 domain and the entire CT domain are retained at the DNA level, but a frame shift deletes these domains at the protein level. The pCR4-TOPO derivative containing the g3 leader fused to the g3 CT domain (see above) and VCSM13 were both cut with BamHI and SnaBI. After isolation from gel, the insert fragment of the pCR4-TOPO derivative, containing the g3 leader, was ligated with the vector fragment of VCSM13, containing a C-terminal portion of the N2 domain and the entire CT domain of g3. The sequence of the insert in the resulting phagemid, named p3-minus, was checked. In this construct, a frame shift occurs at the BamHI site located between the g3 leader and the C-terminal portion of the N2 domain, resulting in the introduction of over 30 stop codons downstream of the BamHI site. The VCSM13-derived helper phage genome p3-minus is depicted schematically in Figure 11.

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CLAIMS

1.   A chimaeric phage having a coat comprising a
mixture of proteins, said mixture comprising a fusion
protein wherein a proteinaceous molecule is fused to a
functional form of a phage coat protein, said mixture
further comprising a mutant form of said phage coat
protein, wherein said mutant form is characterized in
that a phage comprising no wild type phage coat protein
from which said mutant form is derived and having a coat
comprising said mutant form and no copies of said
functional form, is less infectious than a phage
comprising no wild type phage coat protein from which
said mutant form is derived and having a coat comprising
said mutant form and at least one copy of said
functional form.

2.   A chimaeric phage having a coat comprising a
mixture of proteins, said mixture comprising a fusion
protein wherein a proteinaceous molecule is fused to a
functional form of a phage coat protein, said mixture
further comprising a mutant form of said phage coat
protein, said mutant form being impaired in binding to a
host cell receptor.

3.   A chimaeric phage according to claim 1 or 2,
wherein said phage coat protein is the g3 protein.

4.   A chimaeric phage according to claim 3, wherein
said mutant form comprises a mutation in the D1 and/or
the D2 region of said g3 protein.

5.   A chimaeric phage according to claim 4, wherein
said mutation comprises a deletion of substantially all
of said D1 and said D2 region of said g3 protein.

6.   A chimaeric phage according to any one of claims
1-5, comprising a nucleic acid encoding said fusion
protein.

5

7.   A chimaeric phage according to any one of claims
1-6, wherein said chimaeric phage is derived from a M13,
M13K07, VCSM13 or R408 phage.

10   8.   A chimaeric phage according to any one of claims
1-7, wherein said proteinaceous molecule comprises a
peptide, a protein or a part, analogue or derivative
thereof.

15   9.   A chimaeric phage according to any one of claims
1-7, wherein said proteinaceous molecule comprises an
antibody, a Fab fragment, a single chain Fv fragment, a
variable region, a CDR region, an immunoglobulin or a
functional part thereof.

20

10.   A chimaeric phage having a coat comprising a
mixture of proteins, said mixture comprising a fusion
protein wherein a proteinaceous molecule is fused to a
phage coat protein, or to a fragment or derivative
25   thereof, and wherein said fusion protein is functional
so as to render the chimaeric phage infectious, said
mixture further comprising a mutant form of said phage
coat protein, wherein said mutant form is characterized
in that a phage comprising no wild type phage coat
30   protein from which said mutant form is derived and
carrying said mutant form and no copies of said fusion
protein, is less infectious than a phage comprising no
wild type phage coat protein from which said mutant form
is derived and carrying in addition to said mutant form
35   at least one copy of said fusion protein.

11.   A chimaeric phage having a coat comprising a
mixture of proteins, said mixture comprising a fusion
protein wherein a proteinaceous molecule is fused to a
phage coat protein, or to a fragment or derivative
5   thereof, and wherein said fusion protein is functional
so as to render the chimaeric phage infectious, said
mixture further comprising a mutant form of said phage
coat protein, said mutant form being impaired in binding
to a host cell receptor.
10

12.   A chimaeric phage according to claim 10 or 11,
wherein said mutant form is characterized in that a
phage, comprising no wild type phage coat protein from
which said mutant form is derived and carrying said
15   mutant form and no copies of said fusion protein is non-
infectious.

13.   A chimaeric phage according to any one of claims
1-12, wherein said mutant form is further characterized
20   in that a phage having a coat comprising said mutant
form in the presence or absence of copies of said
functional form, is stable.

14.   An infectious phage containing at least one copy
25   of a mutant form of a phage coat protein, wherein said
mutant form has lost the ability to mediate infection of
a natural host by said infectious phage.

15.   A phage collection comprising a chimaeric phage
30   according to any one of claims 1-13 and/or an infectious
phage according to claim 14.

16.   A phage collection according to claim 15, wherein
said phage collection is a phage display library.

35

17.   A phage collection consisting essentially of
chimaeric phages according to any one of claims 1-13 or
of infectious phages according to claim 14.

5   18.   A method for producing a phage particle, said
method comprising the steps of:
         - providing a host cell with a first nucleic acid
encoding a fusion protein, said fusion protein
comprising a proteinaceous molecule fused to a
10   functional form of a phage coat protein;
         - providing said host cell with a second nucleic
acid encoding a mutant form of said phage coat protein,
said mutant form being characterized in that a phage
comprising no wild type phage coat protein from which
15   said mutant form is derived and having a coat comprising
said mutant form and no copies of said functional form,
is less infectious than a phage comprising no wild type
phage coat protein from which said mutant form is
derived and having a coat comprising at least one copy
20   of said functional form, and wherein said host cell
comprises an additional nucleic acid sequence encoding
at least all other proteins or functional equivalents
thereof, that are essential for the assembly of said
phage particle in said host cell; and
25   - culturing said host cell to allow assembly of
said phage particle.

         19.   A method for producing a phage particle, said
method comprising the steps of:
30   - providing a host cell with a first nucleic acid
encoding a fusion protein, said fusion protein
comprising a proteinaceous molecule fused to a
functional form of a phage coat protein;
         - providing said host cell with a second nucleic
35   acid encoding a mutant form of said phage coat protein,

said mutant form being impaired in binding to a host
cell receptor; and

     - culturing said host cell to allow assembly of
said phage particle.

5

20. A method according to claim 18 or 19, wherein
expression of said fusion protein and/or said mutant form
is regulatable by altering the culturing conditions of
said host cell.

10

21. A method according to any one of claims 18-20,
wherein expression of said fusion protein and/or said
mutant form is under the control of a regulatable
promoter.

15

22. A method according to claim 21, wherein said
regulatable promoter comprises the AraC/BAD promoter, the
psp promoter or the lac promoter, or a functional
equivalent thereof.

20

23. A method to any one of claims 18-22, wherein said
additional nucleic acid sequence is provided by a helper
phage to said host cell.

25   24. A method according to claim 23, wherein said helper
phage comprises said second nucleic acid.

25. A method according to any one of claims 18-24,
wherein said fusion protein and said mutant form are
30   encoded by separate nucleic acids each comprising a
unique selection marker.

26. A method according to claim 25, wherein said
separate nucleic acids each comprises a unique origin of
35   replication.

27.   A method according to claim 25 or 26, wherein said
separate nucleic acids each comprises codons that
essentially do not render a homologous recombination
event between said separate nucleic acids.

28.   A method according to any one of claims 18-27,
wherein said phage particle is a chimaeric phage
according to any one of claims 1-13 or an infectious
phage according to claim 14.

29.   A helper phage comprising nucleic acid encoding
phage proteins or functional equivalents thereof that are
essential for the assembly of said helper phage, said
nucleic acid further encoding a mutant form of a phage
coat protein, wherein said mutant form is characterized
in that a phage comprising no wild type phage coat
protein from which said mutant form is derived and having
a coat comprising said mutant form and no copies of a
functional form of said phage coat protein, is less
infectious than a phage comprising no wild type phage
coat protein from which said mutant form is derived and
having a coat comprising at least one copy of said
functional form, wherein said functional form is
characterized in that it renders a phage particle
carrying said functional form in its coat infectious, and
wherein said helper phage does not comprise nucleic acid
encoding said functional form.

30.   A helper phage comprising nucleic acid encoding
phage proteins or functional equivalents thereof that are
essential for the assembly of said helper phage, said
nucleic acid further encoding a mutant form of a phage
coat protein, said mutant form being impaired in binding
to a host cell receptor, and wherein said helper phage
does not comprise nucleic acid encoding a functional form
of said phage coat protein.

31.   A helper phage according to claim 29 or 30, wherein
said phage coat protein is the g3 protein.

32.   A helper phage according to claim 31, wherein said
mutant form comprises a mutation in the D1 and/or the D2
region of said g3 protein.

33.   A helper phage according to claim 32, wherein said
mutation comprises a deletion of substantially all of
said D1 and said D2 region of said g3 protein.

34.   A helper phage according to any one of claims 29-
33, wherein said mutant form is further characterized in
that a phage having a coat comprising said mutant form in
the presence or absence of a copy of said functional
forms, is stable.

35.   A method for producing a helper phage comprising
the steps of:
        - providing a host cell with a first nucleic acid
encoding a functional form of a phage coat protein;
        - providing said host cell with a second nucleic
acid encoding a mutant form of said phage coat protein,
wherein said mutant form is characterized in that a phage
comprising no wild type phage coat protein from which
said mutant form is derived and having a coat comprising
said mutant form and no copies of said functional form,
is less infectious than a phage comprising no wild type
phage coat protein from which said mutant form is derived
and having a coat comprising at least one copy of said
functional form, wherein said host cell comprises an
additional nucleic acid sequence encoding at least all
other proteins or functional equivalents thereof that are
essential for the assembly of said helper phage in said
host cell; and

- culturing said host cell to allow assembly of
said helper phage.

36.   A method for producing a helper phage comprising
the steps of:
- providing a host cell with a first nucleic acid
encoding a functional form of a phage coat protein;
- providing said host cell with a second nucleic
acid encoding a mutant form of a phage coat protein, said
mutant form being impaired in binding to a host cell
receptor, wherein said host cell comprises an additional
nucleic acid sequence encoding at least all other
proteins or functional equivalents thereof that are
essential for the assembly of said helper phage in said
host cell; and
- culturing said host cell to allow assembly of
said helper phage.

37.   A method according to claim 35 or 36, wherein said
other proteins or functional equivalents thereof that are
essential for the assembly of said helper phage in said
host cell are encoded by said second nucleic acid.

38.   A method according to any one of claims 35-37,
wherein expression of said functional form and/or said
mutant form is regulatable by altering the culturing
conditions of said host cell.

39.   A method according to any one of claims 35-38,
wherein expression of said functional form and/or said
mutant form is under the control of a regulatable
promoter.

40.   A method according to claim 39, wherein said
regulatable promoter comprises the AraC/BAD promoter, the

psp promoter or the lac promoter, or a functional
equivalent thereof.

41.   A method according to any one of claims 35-40,
wherein said phage coat protein is the g3 protein.

42.   A method according to claim 41, wherein said mutant
form comprises a mutation in the D1 and/or the D2 region
of said g3 protein.

43.   A method according to claim 42, wherein said
mutation comprises a deletion of substantially all of
said D1 and said D2 region of said g3 protein.

44.   A method according to any one of claims 35-43,
wherein said first nucleic acid and said second nucleic
acid each comprises a unique selection marker.

45.   A method according to any one of claims 35-44,
wherein said first nucleic acid and said second nucleic
acid each comprises a unique origin of replication.

46.   A method according to any one of claims 35-45,
wherein said first nucleic acid and said second nucleic
acid comprise codons that essentially do not render a
homologous recombination event between said first nucleic
acid and said second nucleic acid.

47.   A method according to any one of claims 35-46,
wherein said helper phage is a helper phage according to
any one of claims 29-34.

48.   A method for the enrichment of a first binding pair
member in a repertoire of first binding pair members
selected from the group consisting of an antibody, an
antibody fragment, a single chain Fv fragment, a Fab

fragment, a variable region, a CDR region, an immunoglobulin or a functional part thereof, said first binding pair member being specific for a second binding pair member, comprising the steps of:

5   - contacting a phage collection according to any one of claims 15-17 with material comprising said second binding pair member under conditions allowing specific binding;

           - removing non-specific binders; and

10   - recovering specific binders, said specific binders comprising said first binding pair member.

49.   A method according to claim 48, comprising the additional steps of:

15   - recovering from a phage a DNA sequence encoding said first specific binding pair member;

           - subcloning said DNA sequence in a suitable expression vector;

           - expressing said DNA sequence in a suitable host;

20   and

           - culturing said suitable host under conditions whereby said first specific binding pair member is produced.
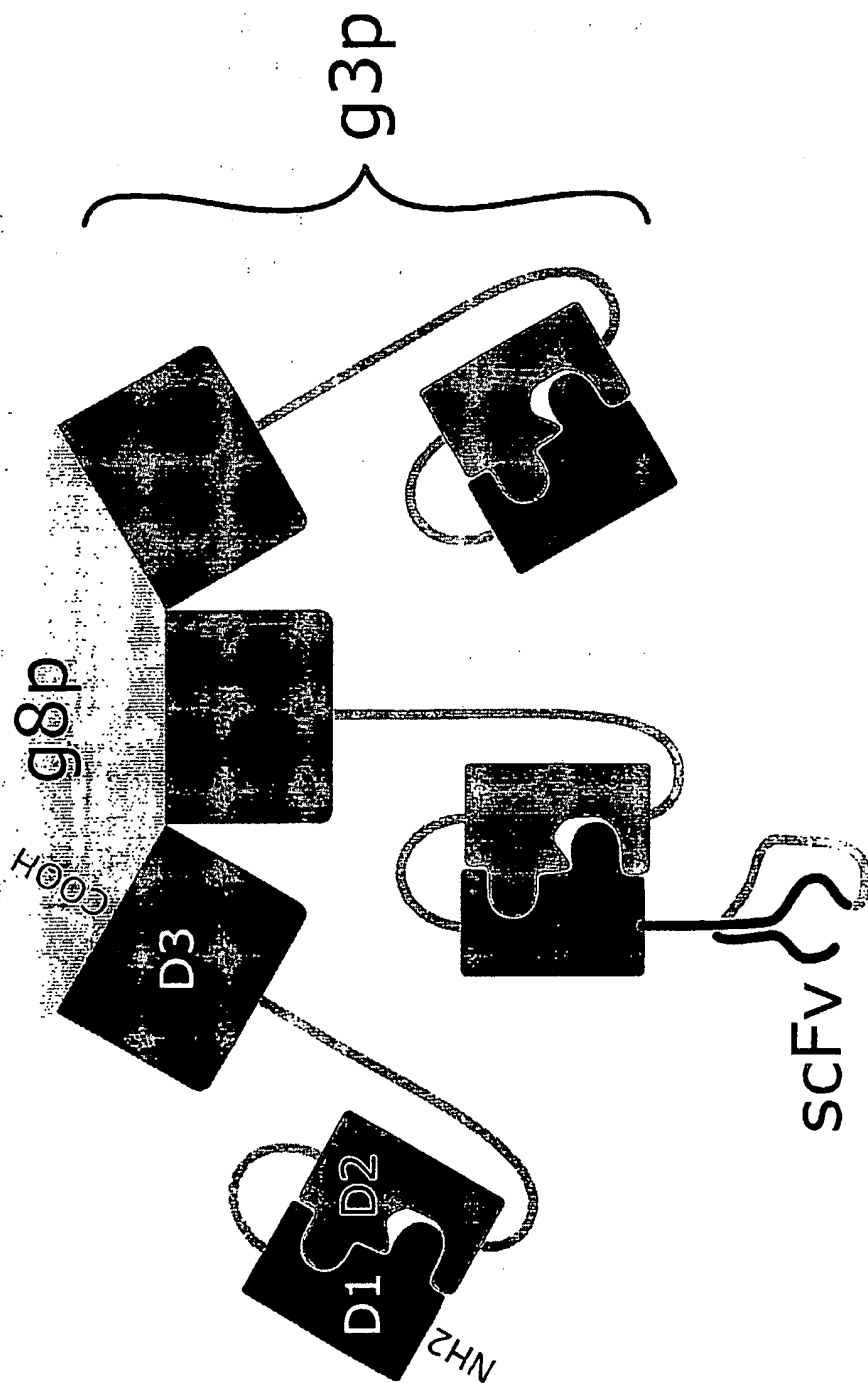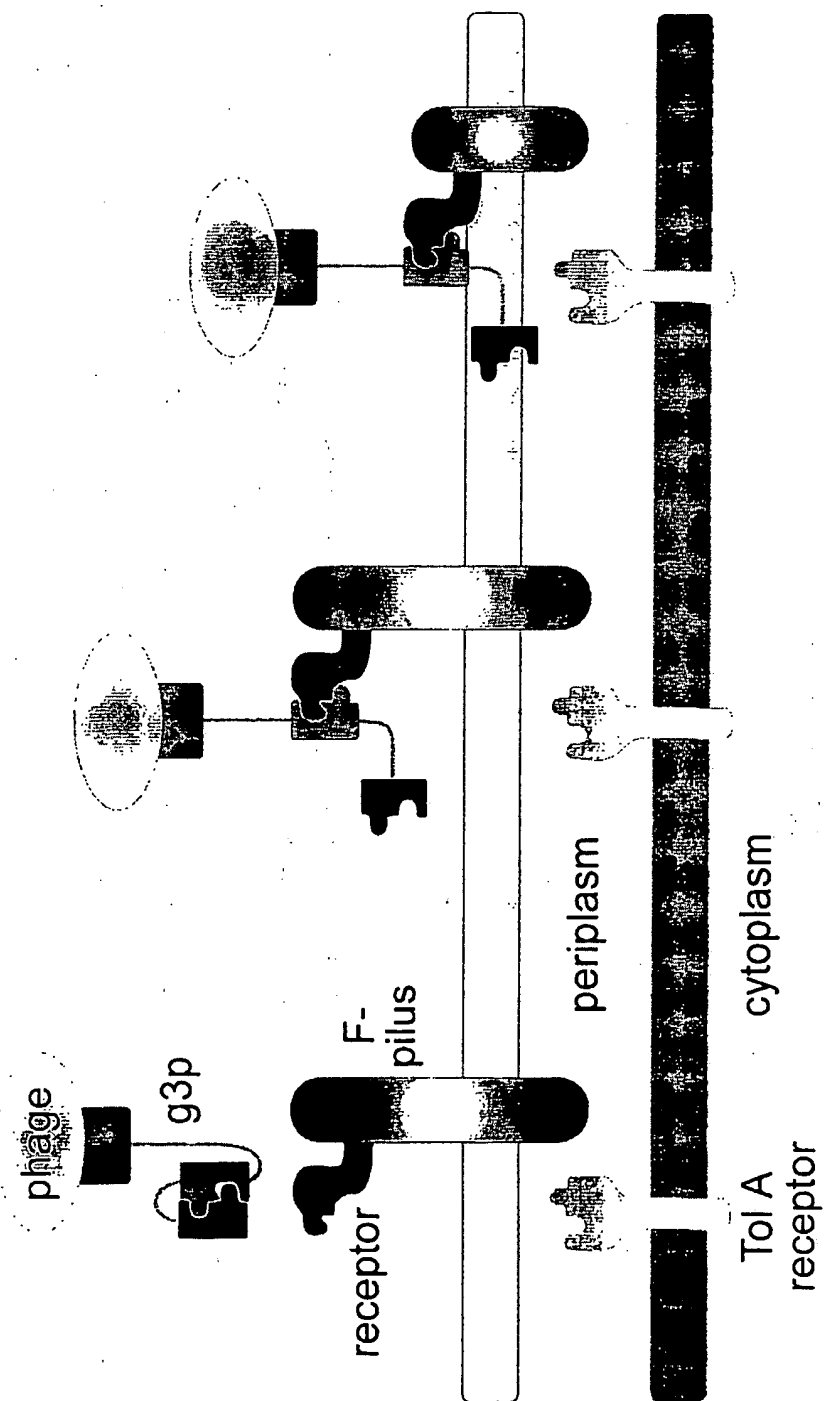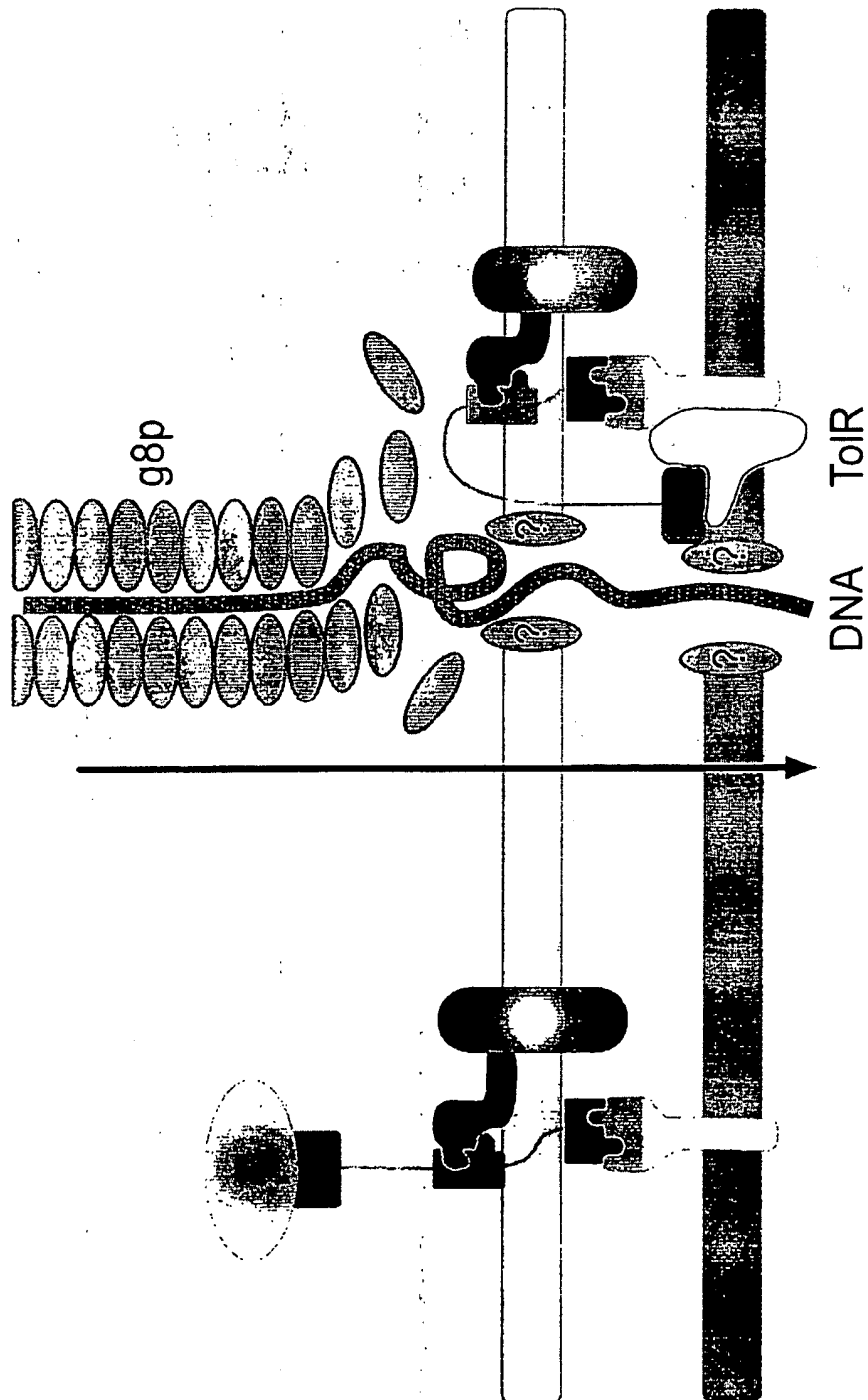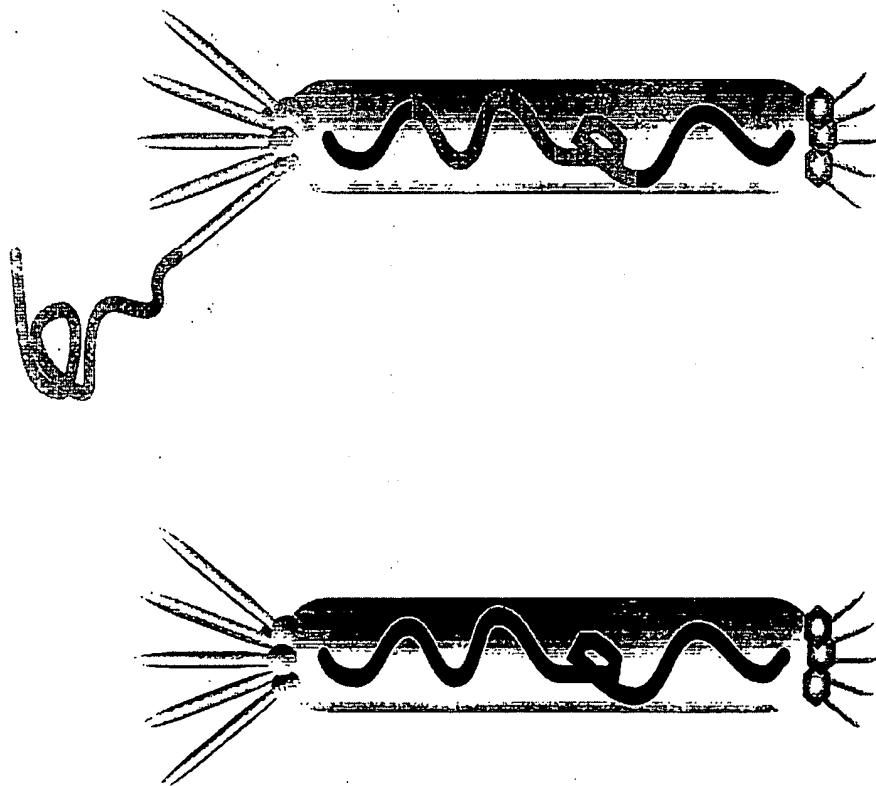
25

Fig. 1

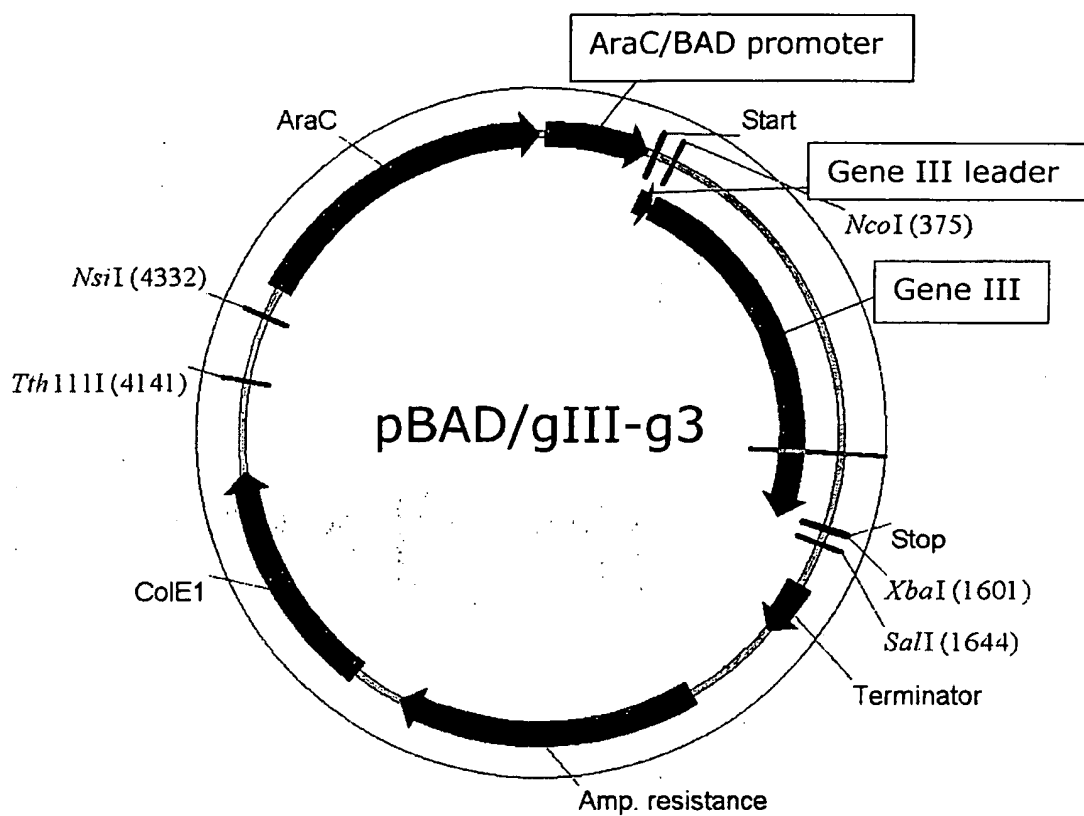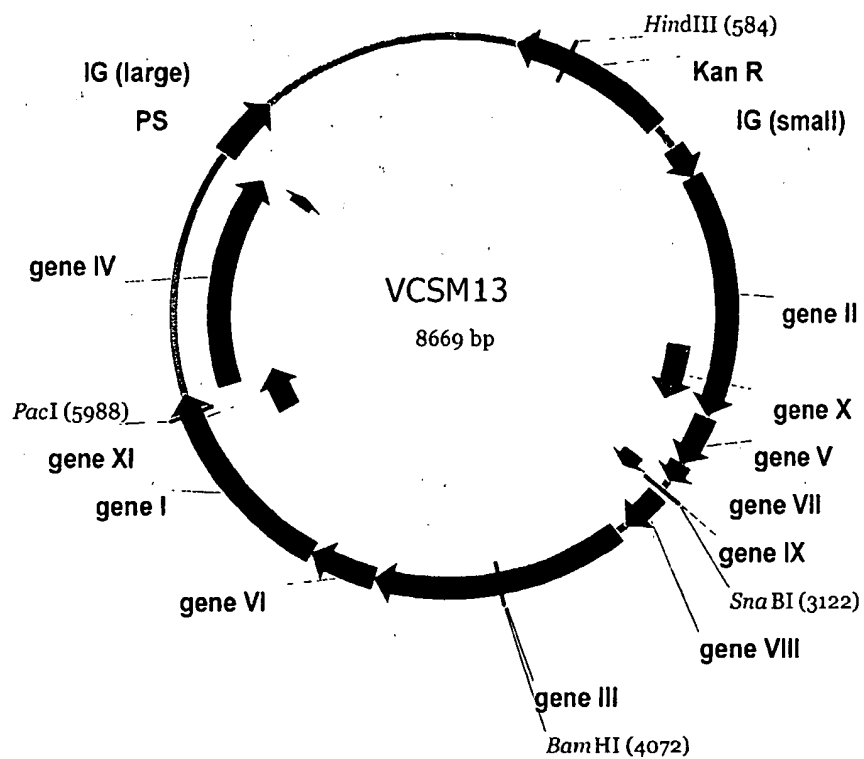Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6A

Fig. 6B

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   1  TGCCTGACTCCCCGTCGTGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCGAATGATACCGGAGACCCACGCTCACCGGCTCCA
 101  GATTTATCAGCAATAAACCAGCCAGCCGATTCGAGCTCGCCCGGGATCGACCAGTTGGTGATTTGAACTTTGCTTTGCCACGGAACGGTCTGCGTTG
 201  TCGGGAAGATGCGTGATCTGATCCTTCAACTCAGCAAAAGTTCGATTTATTCAACAAAGCCGCCTCCCGTCCCGTCAAGTCAGCGTAATGCCTGCCAGTGTTA
 301  CAACCAATTAACCAATTCTGATTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCATACCACATATTTTGAAAAAGC
 401  CGTTTCGTGAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCTGCCAACATCAATACAAC
                                                                     HindIII
                                                                     ~~~~~~
 501  CTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGCTTATGCATTTCTTT
 601  CCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGAGACGAAAT
 701  ACGCGATCGGTCTGTTAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGAT
 801  ATTCTTCTAATACCTGGAATGCTGTTTTCCCGGGGATCGCAGTGGTGAGTAACCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGG
 901  CATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGC
1001  TTCCCATACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCATGTTGGAATTTAATCGCG
1101  GCCTCGAGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATGATATATTTTT
1201  ATCTTGTGCAATGTAACATCAGAGATTTTGAAACACAACGTGGCTTTCCCCCCCCTGCAGTCTCCGGCTATTCTTTTGATTTATAAGGGATTTT
1301  TGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAATATTAACGAATTTAACAAATATTAACGTTTACAATTTAAATATTTGCTTA
1401  TACAATCTTCCTGTTTTTGGGGCTTTTCTTATTATCAACCGGGTACATAATGTTTTACGATTTCATGATTGCACGGTTGAATATCATG
1501  TCCAGACTCTCAGGCAATGACCTGATAGCCTTTCGTGTAGAACCCTTTGAATCTTGAATCCTTTTCTCACCCTTTGAAAAGTATTACAGGGTTCTAAAAA
1601  TTGATGGTGATTTGCGTTGACTGTCTCCGGCCTTTCGTTGAATAAAGGCTTCCTCCTGCCGTGTGTGAAATAAAGGCTTCTTAAAATATATGGGTTCTGAGGCTTTA
1701  TTTTTATCCTTGCGTTGTTTGCTAATTCTTTGCCTTGCCTGTATGATTTATTGGATGTTAATGCTACTACTATTAGTAGAATTACTACTGTTCAGCTCCGCGCCCC
1801  TTGCTTAATTTTGCTAATTCTTTGCCTTGCCTGTATGATTTATTGGATGTTAATGCTACTACTATTAGTAGAATTGATGCCAGAATTGGGACAACTGTTAC
1901  CAAATGAAAATATAGCTAAACAGGTTATTGACCATTTGCAAATTCTTAAAACCTGATGGCTACTACTGTTGAGCGTTGGCTTTATACTGGTAAGAATTTGTATAAGC
2001  ATGGAATGAAACTTCCAGACACCGTACTTTAGTTGCATATTCTAATCCTGACTGTTGAGCGTTGGCTTTATACTGGTAAGAATGTCGTAACATAGTTGAGGCTACTACTGTTGAGC
2101  GATATTTGAAGTCTTTCGGGCTTCCTCTTAATCTTTTTGATGCAATCGTTTTGACTATAATAGTCAGGGTAAAGACCTGATTTTTGATTTATG
2201  GTCATTCTCGTTTTCTGAACTGTTTAAAGCATTTGAGGGGGATTCAATGAATATTTATGACGATTCCGCAGTATTGGACGCTATCCAGTCTAAACATTT
2301  ACTATTACCCCCTCTGGCAAAACTTCTTTTGCAAAAGCCTCTCGCTATTTTGGTTTTTTATCGTCGTCTGGTAAACGAGGGTTATGATAGTGTTGCTCTTA
2401  CTATGCCTCGTAATTCCTTTTGGCGTTATGTATCTGCATTAGTTGAATGTGGTATTCCTAAATCTCAACGGCAGAGGCATTCACAATGATTA
2501  TCGGTTAGTTCGTTTATTAACGTAGATTTTTCTTCCCAAATGAGCCAGTTCCATAGGATGAATGGGCAAGCCTTATTCACTGAATGAGCAGCTTTGTTACGTTGA
2601  AAGTTGAAATTAAACCATCTCAAGCCCAATTACTTACTACTGCTCTGCGCCCTCTGGTCTGTGCGCCTGTCAGCCTAAGTAACATGGAGCAGGTCGCGGATTTCGACACAATTATCAGGCGATG
2701  TTTGGGTAATGAATATCCGGTTCTTGTCAAGATTACTCTTGATGAAGGTCAGCCAGCCTATGCGCCTGGTCTGTACACCGTTCATCTGTCCTCTTTCAAA
2801  GTTGGTCAGTTCGGTTCCCTTATGATTGACCGTCTGCGCCTCGTTCCGGCTAAGTAACATGGAGCAGGTCGCGGATTTCGACACAATTATCAGGCGATG
2901  ATACAAATCTCCGTTGTACTTTGTTTCGCGCTTGGTATAATCGCTGGGGGTCAAAGATGAGTGTTTTAGTGTATTCTTTCGCCTCTTTCGTTTTAGGTTG
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Fig. 6B, contd.

SnaBI

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3101 GTGCCTTCGTAGTGGCATTACGTATTTTACCCGTTTAATGGAAACTTCCTCATGAAAAAGTCTTTAGTCCTCAAAGCCTCTGTGTAGCCGGTGCTACCCTCG
3201 TTCCGATGCTGTCTTTCGCTGCTGAGGTGACGATCCCGCAAAAGCGGCCTTTAACTCCCTGCAAGCCTCAGCGACCGAATATCGGTTATGCGTGGGC
3301 GATGGTTGTTGTCATTGTGCGGCCAACTATCGGTATCGGTTCAAGCTGTTAAGAAATTCACCTCGAAAGCAAGCTGATACAACCGATACAATTAAAGGCTCCTTT
                                                       V  K  K  L  F  A  I  P  L  V  V  P  F  Y  S  H  S  A  E  T  V  E  S
3401 TGGAGCCTTTTTTTTTGGAGATTTCAACGTGAAAAAATATTATTCGCAATTCCTTAGTTGTTCCTTCATTCCGCTGAAACTGTTGAAAG
         C  L  A  K  P  H  T  E  N  S  F  T  N  V  W  K  D  D  K  T  L  D  R  Y  A  N  Y  E  G  C  L  W  N
3501 TTGTTTAGCAAAAAACCCATACAGAAAATTCATTTACTAACGTCTGGAAAGACGACAAAACTTTAGATCGTTACGCTAACTATGAGGGCTGTCTGTGGAAT
        A  T  G  V  V  V  C  T  G  D  E  T  Q  C  Y  G  T  W  V  P  I  G  L  A  I  P  E  N  E  G  G  S  E
3601 GCTACAGGCGGTTGTAGTTTGTACGGCGAAACTCAGTGTTACGGTACGGTACCATGGGCTTGCTATCCCTGAAAATGAGGGTGGTGGCTCTG
          G  G  G  S  E  G  G  G  S  E  G  G  G  T  K  P  P  E  Y  G  D  T  P  I  P  G  Y  T  Y  I  N  P  L
3701 AGGGTGGCCGGTTCTGAGGGTGGCGGTTCTGAGGGTGGCGGTTCTGAGTACGGTGATACAACCTCCTGGTTATACTTATATCAACCCTCT
          D  G  T  Y  P  P  P  G  T  E  Q  N  P  A  N  P  N  P  S  L  E  E  S  Q  P  L  N  T  F  M  F  Q  N  N
3801 CGACGGCACTTATCCGCCTGCCTGTGACTGAGCAAAACCCCGCTAATCCTAATCCTTCTCTTGAGGAGTCTCAGCCTCTTAATACTTTCATGTTTCAGAATAAT
          R  F  R  N  R  Q  G  A  L  T  V  Y  T  G  T  V  T  G  V  T  D  P  V  K  T  Y  Y  Q  Y  T  P  V  S  S
3901 AGGTTCCGAAATAGGCAGGGGCATTAACTGTTTATACGGGGACACTGTTACTCAAGGCACTGACCCCGTTAAAACTTATTACCAGTACACTCCTGTATCAT
                                                    BamHI
          K  A  M  Y  D  A  Y  W  N  G  K  F  R  D  C  A  F  H  S  G  F  N  E  D  P  F  V  C  E  Y  Q  G  Q
4001 CAAAAGCCATGTATGACGCTTACTGGAACGGTAAAATTCAGAGACTGCGCTTTCCATTCTGGCTTTAATGAGGATCCATTCGTTTGTGAATATCAAGGCCA
          S  S  D  L  P  Q  P  P  V  N  A  G  G  G  S  E  G  G  G  S  E  G  G  G  S
4101 ATCGTCTGACCTGCCTCAACCTCCTGTCAATGCTGGCGGCGGCTCTGGTGGTGGTTCTGAGGGTGGCGGCTCTGAGGGTGGCGGTTCT
          E  G  G  G  S  E  G  G  G  S  E  G  G  G  S  G  D  F  D  Y  E  K  M  A  N  A  N  K  G  A  M  T  E
4201 GAGGGTGGCGGCTCTGAGGGAGGCGGTTCCGGTGGTGGCTCTGGTTCCGGTGATTTTGATTATGAAAAGATGGCAAACGCTAATAAGGGGGCTATGACCG
          N  A  D  E  N  A  L  Q  S  D  A  K  G  K  L  D  S  V  A  T  D  Y  G  A  A  I  D  G  F  I  G  D  V
4301 AAAATGCCGATGAAAACGCGCTACAGTCTGACGCTGATTCTGTCGCTACTGATTACGGTGCTGCTATCGATGGTTTCATTGGTGACGT
          S  G  L  A  N  G  N  G  A  T  G  D  F  A  G  S  N  S  Q  M  A  Q  V  G  D  G  D  N  S  P  L  M  N
4401 TTCCGGCCTTGCTAATGGTAATGGTGCTACTGGTGATTTTGCTGGCTCTAATTCCCAAATGGTTGTCTTCGGCTCTAATTCACCTTTAATGAAT
          N  F  R  Q  Y  L  P  S  L  P  Q  S  V  E  C  R  P  F  V  F  G  A  G  K  P  Y  E  F  S  I  D  C  D  K
4501 AATTTCCGTCAATATTTACCTTCGCCTCCCTCAATCGGTTGAATGTCGCCCTTTTGTCTTTGGCGCTGGTAAACCATATGAATTTTCTATTGATTGTGACA
          I  N  L  F  R  G  V  F  A  F  L  L  Y  V  A  T  F  M  Y  V  F  S  T  F  A  N  I  L  R  N  K  E  S
4601 AAATAAACTTATTCCGTGGTGTCTTTGCGTTTCTTTTATATGTTGCCACCTTTATGTATGTATTTTCTACGTTTGCTAACATACTGCGTAATAAGGAGTC
                     *
4701 TTAATCATGCCAGTTCTTTTGGGTATTCCGTTATTATTGCGTTTCCTCGGTTTCCTTCTGGTAACTTTGTTCGGCTATCTGCTTACTTTTCTTAAAAGG
4801 GCTTCGGTAAGATAGCTATTGCTATTTCATTGTTTCTTGCTCTTATTATTGGGCTTAACTCTCTGTTATCTGTATAAATAGGCCTCGATATAGGCCTCAATT
4901 ACCCCTGACTTTGTTCAGGGTGTTCAGTTAATTCTCCCGTCAATGCGTCCTAATGCGTTTGTTATTCTCTGAAAGGCTGCTATTTTCATTTT
5001 GACGTTAAACAAAAATCGTTTCTTATTTGGATTGGGTGCAAAATAAGTGCAACTAATCTTGATTTAAGGCTTCAAAAACCTCCCGCAAGTCGGTTAGC
5101 GTTGGTAAGATTCAGGATAAATTGTAGCTGGGTGCAAAATAGCAACTAATCTTGATTTAAGGCTTCAAAACCTCCCGCAAGTCGGGTAGGCTTGCTAAAA
5201 CGCCTCGCGTTCTTAGAATAGTGCGGTACTTGGTTTAATACCCGTTCTTGGAATGATAAGGAAAGACAGCCGATTATTGATTGGTTTCTACATGCTCGTAGC
5301 TGTTTCCTGATGAGTGCGGTACTTGGTTTAATACCCGTTTTATTGGTCTATTTCTTATTATTGTTCACATGTTGTTTATTGTCGTCGGGACAGAA
5401 TGGGATATTATTTTTCTTTGTCGGTACTTTATATTCTCTTATTACTGGCTCGAAATGCCTCTGCCTAAATTACATGATGAATTAAATTACATGTGTTAAATATGGCGATTCTCA
5501 TTACTTTACCTTTTGTCGGTACTTTATATTCTCTTATTACTGGCTCGAAATGCCTCTGCCTAAATTACATGATGAATTAAATTACATGTGTTAAATATGGCGATTCTCA
5601 ATTAAGCCCTACTGTTGAGCGTTGGCTTTATCACACGGTCGGTATTTCAAACCATTAAAATTTGTATAAACCATTAAAATTAACTAAATATATTGAATATTTT
5701 TATTTCTTATTATCACGCGTATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAACCAAATAGCAACCAGTT
5801 CTCGCGTTCTTTGTCTTGCGATTGGATTTGCATCAGCATTTACATATAGTTATATAACCCAACCTAAGCCGGAGTTAAAAAGGTAGTCTCTCAGACCTA
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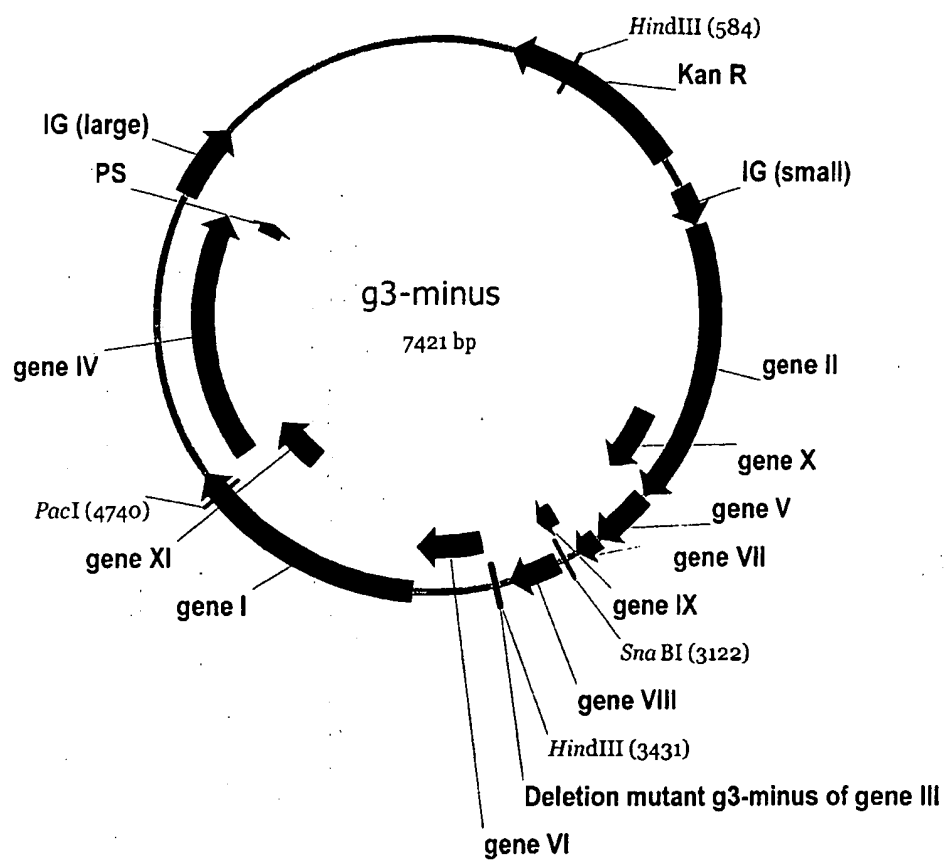
Fig. 6B, contd.

PacI

```
5901  TGATTTTGATAAATTCACTATTGACTCTTCTCAGCGTCTTAATCTAAGCTATCGCTATGTTTCAAGGATTCTTCAAGGATTCTTCAAGGAAAATTAATTAATAGCGACGAT
6001  TTACAGAAGCAAGGTTATTCACTCACATATTGATTTATGTACTGTTTCCATTAAAAAAGGTAATTCAAATGAAATTGTTAAATGTTAATTAATTTGTT
6101  TTCTTGATGTTGTTTCATCATCTTCTTTGCTCAGGTAATTGAAATGAATAATTCGCCTCTGCCGATTTGTAACTTGTATTCAAAGCAATCAGGCG
6201  AATCCGTTATTGTTTCTCCCGATGTAAAAGGTACTGTATATTCATCTGACGTTAAAACCTGAAAATCTGAAAAATCTACGCAATTCTTATTTCTGTTTTACG
6301  TGCAAATAATTTTGATATGGTAGGTTCTAACCCTTCCATTATTCAGAAGTATAATCAAACAATCAGGATTATATTGATGAATTGCCATCATCTGATAAT
6401  CAGGAATATGATGATAATTCCGCTCCTTCTGGTGGTTTCTTTGTTCCCGAAAAATGATAAATCTCAAACTTTTAAAATTAATAACGTTCGGGCAAAGG
6501  ATTTAATACGAGTTGTCGAATTGTTTGTAAAGTCTAATACTTCTAAATCCTCAAATGTTATTATCTATTCTATTAGTTGTTAGTGCTCC
6601  TAAAGATATTTTAGATAACCTTCCTCAATTCCTTTCAACTGTTGATTTGCCAACTGACCAGATATTGATTGAGGGTTGATATTTGATGTTCAGCAAGGT
6701  GATGCTTTAGATTTTTCATTTGCTGGGGGGGGGGGGGTGGGGGTTGCAGGCGGTGTAATACTGACCGCTCACCCTCTGTTTTATCTTCTGCTGGTG
6801  GTTCGTTCGGTATTTTTAATGGCCGATGTTTTAGGGCTATCAGTTCGCGCATTAAAGACTAATAAGCATTCAAAAATATTGTCTGTGCCAATGTAAATAATCCATTTCAG
6901  GCTTTCAGTTCAGGAAGGGTTCTATCTCTGTTGGCCAGAATGTCCCTTTATTACTGGTCGTGTGACTGGTGAATCTGCGTAATATGTTCTGGATATTACCAGGCCGATAGTT
7001  ACGATTGAGCGTCTACTCAGGCAAGTGTAGGTTATTACTAATCAAAGAAGTATTGCTACAACGGTTAATTTGCGTGATGGACAGACTCTTTTACTCGGTGGCCT
7101  TGAGTTCTTCTACTGGGCAAGTGATGTTATTACTAATCAAAGAAGTATTGCTACAACGGTTAATTGGCTCCTGTTTAGCTCCCGCTCGATTCTAACGAG
7201  CACTGATTATAAAAACACTTCTCAGGATTCTGGCGTTCCTGTCTAAAATCCCTTTAATCGGGCCCTGTTAGCGCGGGGCAGCGTGACCGGCT
7301  GAAAGCACGTTATACGTCGTCGTCAAAGCAACCATAGTACGCCCTAGTAGCCCTCCTTTCGCTTTCTCCTTCCTCCGCCGGCTTTCCCCACGTTCAAGCTCAAATCGGGGGCTCC
7401  ACACTTGCCAGCGCCCTAGCGCCCGGCTCCTTTCGCTTTCTCCTTCCTCCGCCACCTGATTTGGGTGATGGTTCACGTAGTGGGCCATGCCCTGATAGACGGTTTT
7501  CTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTTGGGTGATGGTTCACGTAGTGGGCCATGCCCTGATAGACGGTTTT
7601  TCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGAACAACACTCAACCTATCTCGGGACGGATCGCTTCATGTGGC
7701  AGGAGAAAAAGGCTGCACCGGTGCGTCAGCAGAATAGTGTATACAGGATATATTCCGCTTCCTGCCTCACTGACTCCGGTCGTTCGACTGC
7801  GGCGAGCGGGAAATGGCTTACGACACGGGGCGGGAGATTCCTGGAAGATGCCAGGAGAATACTTAACAGGAGAGTGAGAGGGCCCGCGCAAAGCCGTTTTC
7901  CATAGGCTCCCGCCCCCTGACAAGCATCACGAAATCTGACGCTCAAATCAGTGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTG
8001  GCGGGTCCCTGCGCGTCTCGTGTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGTCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCC
8101  CCGGGTAGGCAGTTCGCTCCAAGCTGGACTGTATGCACGAACCCCCGTTCAGTCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCC
8201  GGAAAGACATGCAAAAGCACCACTGGCCAGCCACTGGTAATTGATTTAGAGGAGTAGTCTTGAAGTCATGCGCCGGTTAAGCTAAACTGAAAAGAC
8301  AAGTTTTGGTGACTGGCGCTGCCTCCTCCAAGCCAGTTACCTCGGTTCAAGAGATCATCTTATTAAGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGA
8401  TTTTGGTCATGAGATTATCAAAAAGGATCTTCAACGTCTAGGATCTTCAGATCCTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTATATGAGTAAAACTTGGTC
8501  TTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCTCAGCGATCTGTCTATTTCGTTCATCCATAGT
8601  TGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGT
```

Fig. 6C

G3-minus protein

GGTTATGCGTGGGCGATGGTTGTTGTCATTGTCGGCGCAACTATCGGTATCAAGCTGTTTAAGAAATTCACCTCG
AAAGCAAGCTGATAAACCGATACAATTAAAGGCTCCTTTTGGAGCCTTTTTTTTTGGAGATTTTCAAC**AAGCTTC**
TGCGTAATAAGGAGTCT_TA_ATCATGCCAGTTCTTTTGGGTATTCCGTTATTATTGCGTTTCCTCGGTTTCCTTCT
GGTAACTTTGTTCGGCTATCTGCTAACTTTTCTTAAAAAGG

Fig. 6D
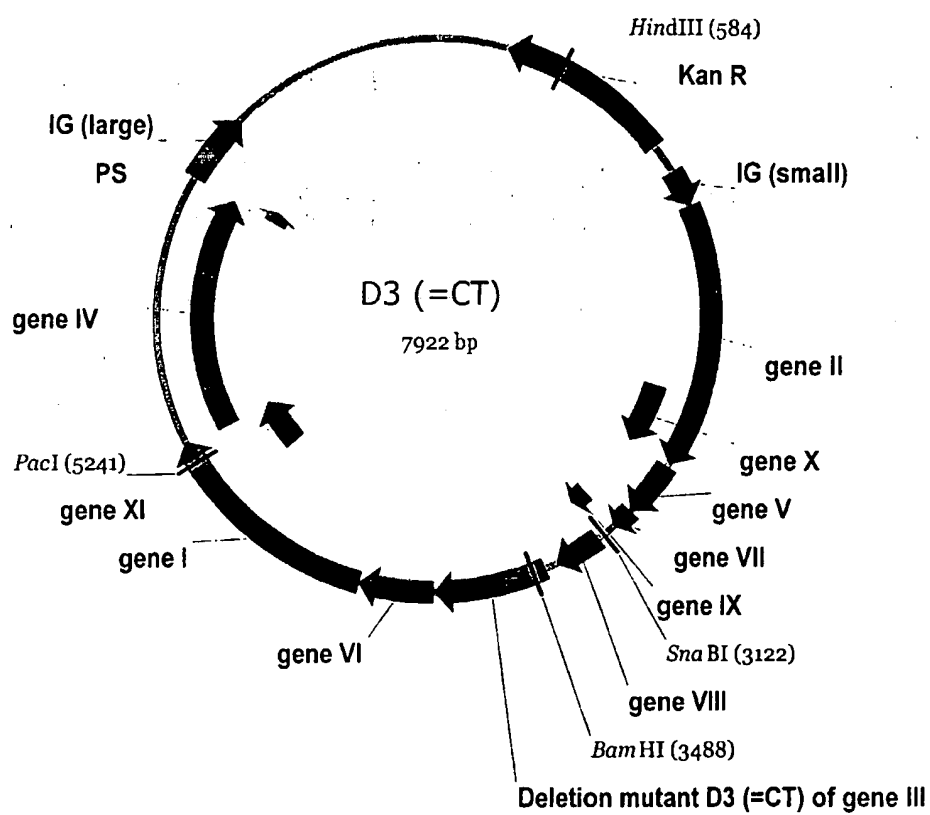
Fig. 7A

D3 domain of g3 Protein

```
TTGACTCCCTGCAAGCCTCAGCGACCGAATATATCGGTTATGCGTGGGCGATGGTTGTTGTCATTGTCGGCGCAA
CTATCGGTATCAAGCTGTTTAAGAAATTCACCTCGAAAGCAAGCTGATAAACCGATACAATTAAAGGCTCCTTTT
GGAGCCTTTTTTTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTTGTTCCTTTCTATTC
TCACTCCGCTGGATCCTCTGGTTCCGGTGATTTTGATTATGAAAATATGGCAAACGCTAATAAGGGGGCTATGAC
CGAAAATGCCGATGAAAACGCGCTACAGTCTGACGCTAAAGGCAAACTTGATTCTGTCGCTACTGATTACGGTGC
TGCTATCGACGGTTTCATTGGTGACGTTTCCGGCCTTGCTAATGGTAATGGTGCTACTGGTGATTTTGCTGGCTC
TAATTCCCAAATGGCTCAAGTCGGTGACGGTGATAATTCACCTTTAATGAATAATTTCCGTCAATATTTACCTTC
CCTTCCTCAATCGGTTGAATGTCGCCCTTTTGTCTTTCGCGCTGGTAAACCATATGAATTTTCTATTGATTGTGA
CAAAATAAACTTATTCCGTGGTGTCTTTGCGTTTCTTTTATATGTTGCCACCTTTATGTATGTATTTTCGACGTT
TGCTAACATACTGCGTAATAAGGAGTCTTAATCATGCCAGTTCTTTTGGGTATTCCGTTATTATTGCGTTTCCTC
GGTTTCCTTCTGGTAACTTTGTTCGGCTATCTGCTAACTTTTCTTAAAAAGGGCTTCGGTAAGATAGCTATTGCT
ATTTCAT
```

Fig. 7B

A	GCG GCA GCC GCT
C	TGC TGT
D	GAT GAC
E	GAA GAG
F	TTT TTC
G	GGT GGC
H	CAT CAC
I	ATT ATC ATG
K	AAA
L	CTG
M	ATG
N	AAC AAT
P	CCG
Q	CAG
R	CGT CGC
S	AGC TCT
T	ACC ACG
V	GTG GTT GTC
W	TGG
Y	TAT TAC
STOP	TAA TGA

Fig. 8
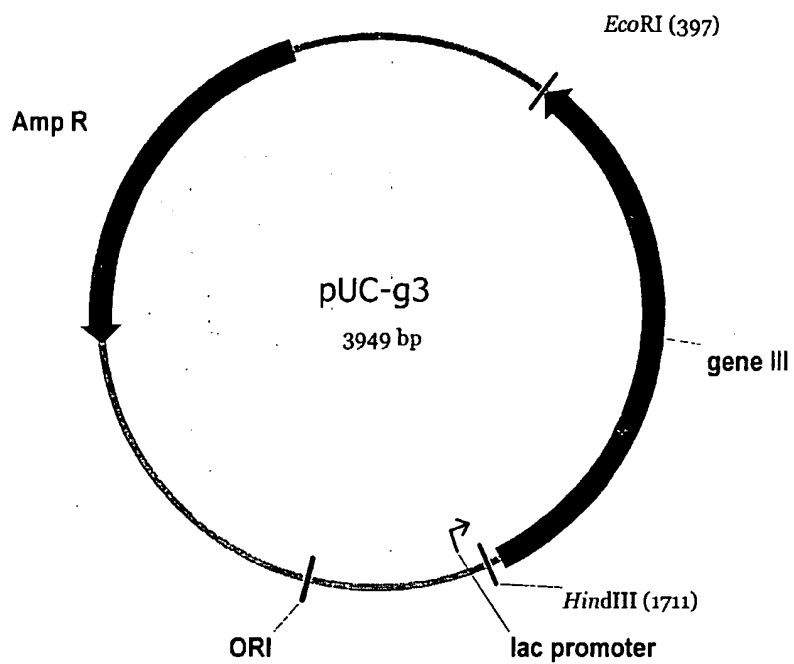
Fig. 9
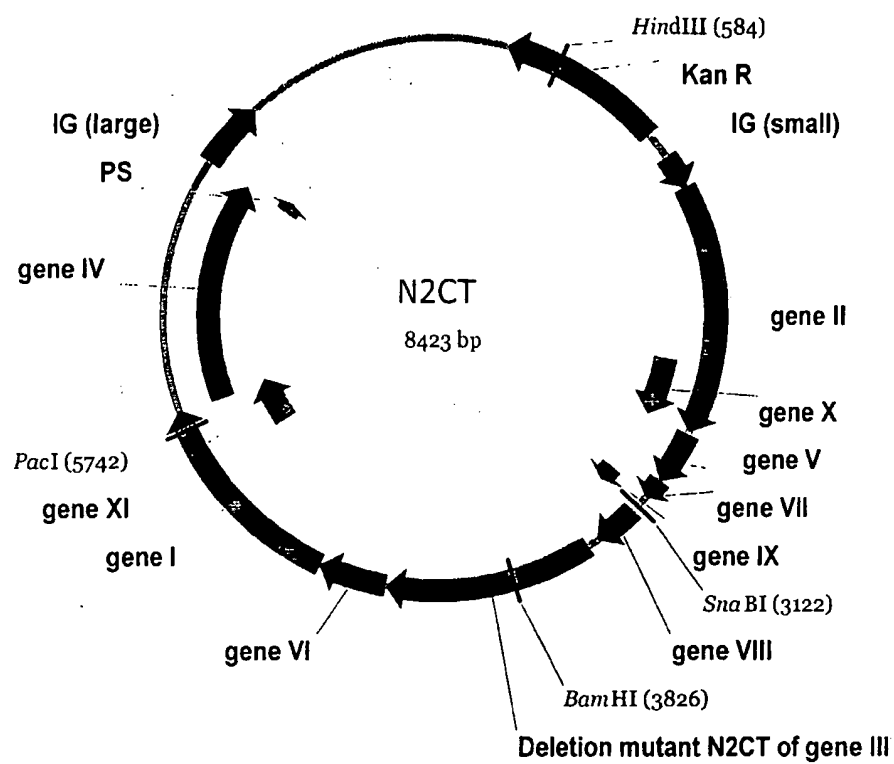
Fig. 10
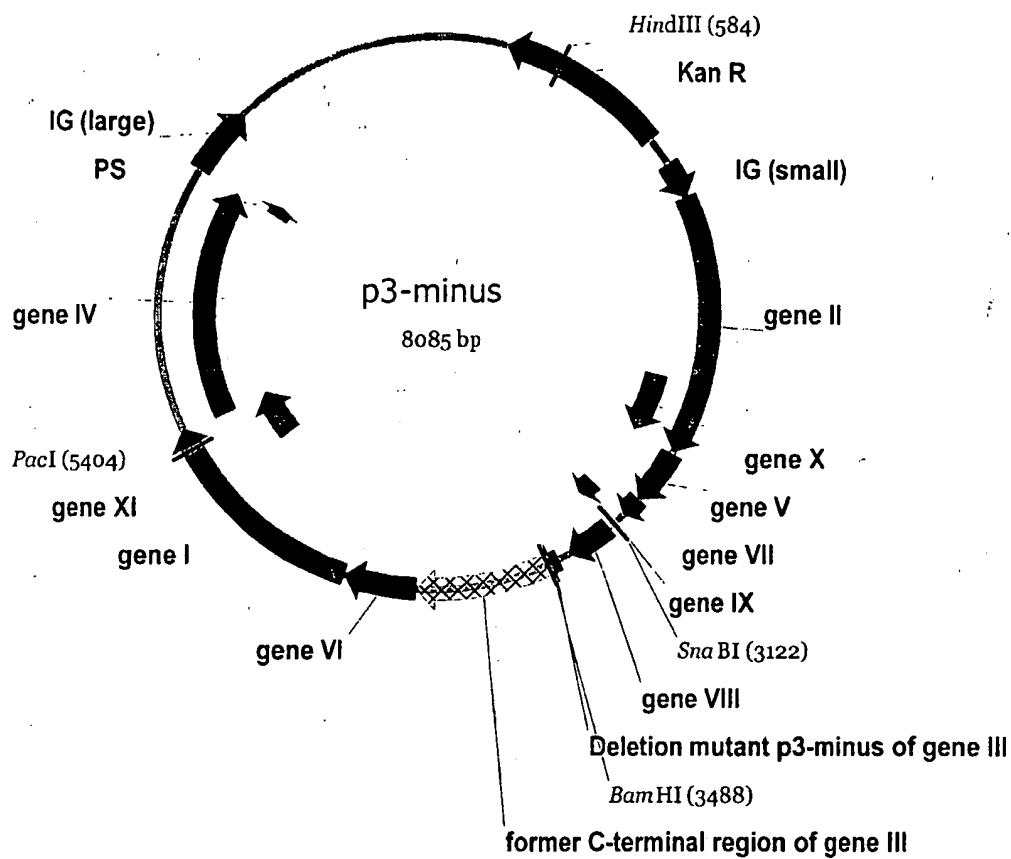
Fig. 11

INTERNATIONAL SEARCH REPORT

WO 02/103012

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C12N15/10   G01N33/68   C12N7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C12N   G01N

Documentation searched other than minimum documentation to the extent that such documents are included In the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KREBBER CLAUS ET AL: "Selectively-infective phage (SIP): A mechanistic dissection of a novel in vivo selection for protein-ligand interactions" JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 268, no. 3, 1997, pages 607-618, XP002151869 ISSN: 0022-2836 see whole doc. esp. fig. 1 and p.615,2.col., 3.par. --- -/--	1-49

[X] Further documents are listed in the continuation of box C.   [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 October 2002	25/11/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mueller, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	NILSSON N. ET AL.,: "The phage infection process: a functional role for the distal linker region of bacteriophage protein 3" J. VIROLOGY, vol. 74, no. 9, May 2000 (2000-05), pages 4229-4235, XP002183102 see whole doc. esp. Figure 1 and discussion	1-49
X	CHATELLIER J. ET AL.,: "Interdomain interaction within the gene 3 protein of filamentous phage" FEBS LETTERS, vol. 463, no. 3, - 1999 pages 371-374, XP002183103 cited in the application see whole doc., esp. abstract, Fig 1,	1-49
X	RONDOT S. ET AL.,: "A helper phage to improve single-chain antibody presentation in phage display" NATURE BIOTECH, vol. 19, no. 1, January 2001 (2001-01), pages 75-78, XP002183104 see whole doc. esp. abstract and figures	1-49
A	DUENAS M ET AL: "CLONAL SELECTION AND AMPLIFICATION OF PHAGE DISPLAYED ANTIBODIES BY LINKING ANTIGEN RECOGNITION AND PHAGE REPLICATION" BIO/TECHNOLOGY, NATURE PUBLISHING CO. NEW YORK, US, vol. 12, 1 October 1994 (1994-10-01), pages 999-1002, XP000575847 ISSN: 0733-222X see whole doc.,esp.figure 1 and discussion	
A	RAKONJAC J. ET AL.,: "Filamentous phage infection-mediated gene expression: construction and propagation of the gIII deletion mutant helper phage R408d3" GENE, vol. 198, - 1997 pages 99-103, XP002183105 cited in the application see whole doc., esp. experimental and discussion, p.100, 1.col and 2.col.	

-/--

Intern   Application No

PCT/NL 0PCT/NL02/00391

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	KRUIF DE J ET AL: "SELECTION AND APPLICATION OF HUMAN SINGLE CHAIN FV ANTIBODY FRAGMENTS FROM A SEMI-SYNTHETIC PHAGE ANTOBODY DISPLEY LIBRARY WITHDESIGNED CDR3 REGIONS" JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 248, no. 1, 21 April 1995 (1995-04-21), pages 97-105, XP000646544 ISSN: 0022-2836 cited in the application the whole document	